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(54) Title: DIAGNOSTIC METHOD FOR BRAIN DAMAGE-RELATED DISORDERS

(57) Abstract: A brain damage-related disorder is diagnosed in a subject by detecting at least one polypeptide, or a variant or mutant there« selected from A-FABP, E-FABP, PGP 9.5, GFAP, Prostaglandin D synthase, Neuromodulin, Neurofflament L, Calcyphosine, RNA binding regulatory subunit, Ubiquitin fusion degradation protein 1 homolog, Nucleoside diphosphate Idnase A, Glutathione S tranférase P, Cathepsin D, DJ-1 protein, Peroxiredoxin 5 and Peptidyl-prolyl cis-trans isomerase A (Cyclophilin A) in a sample of body fluid taken from the subject.

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DIAGNOSTIC METHOD FOR BRAIN DAMAGE-RELATED DISORDERS

BACKGROUND OF THE INVENTION

Field of the invention

This invention relates to a diagnostic method for brain damage-related disorders.

No biological marker is currently available for the routine diagnosis of brain damage-related disorders including cerebrovascular, dementia and neurodegenerative diseases. This invention relates to the use of cerebrospinal fluid from deceased patients as a model for the discovery of brain damage-related disorder markers, and to the use of such markers in diagnosis.

Description of the related art

Over the last two decades, a number of biological markers (biomarkers) have been studied in the cerebrospinal fluid (CSF) and serum of patients with brain damage-related disorders, including creatine kinase-BB [1], lactate dehydrogenase [2], myelin basic protein [3], S100 protein [4], neuron-specific enolase (NSE) [5], glial fibrillary acidic protein [6] and tau [7]. Most of them have not proved useful indicators of the extent of brain damage and accurate predictors of clinical status and functional outcome. In fact, the diagnostic value of biomarkers for brain damage-related disorders has been hampered by their late appearance and a delayed peak after the damage event, their poor sensitivity and specificity, and the limited understanding of the mechanisms governing the release of these molecules into the CSF and ultimately in the blood. As a result of these limitations, the use of brain damage-related disorder biomarkers is currently limited to research settings and none has been recommended for routine assessment [8].

WO 01/42793 relates to a diagnostic assay for stroke in which the concentration of heart or brain fatty acid binding protein (H-FABP or B-FABP) is determined in a sample of body fluid.

SUMMARY OF THE INVENTION

Ideally, a biomarker for the diagnosis, monitoring and prognosis of brain damage-related disorders should include at least the following characteristics: (1) it should be brain-specific; (2) because of obvious difficulties to obtain CSF samples in patients, detection in serum is highly desirable; (3) it should appear very early; (4) its peak level, alternatively the area under the curve of sequential concentrations, should reflect the extent of brain damage; finally (5) it should be indicative of functional outcome. We demonstrate here new brain damage-related disorder biomarkers and provide a comparison with S100 and NSE, the two molecules, which have been most extensively assessed for this purpose.

We describe how proteins have been identified as new diagnostic biomarkers for brain damage-related disorders using a proteomics-based analysis of CSF from deceased patients as a model of massive brain damage. And we report as an example on results obtained after serum FABP levels have been sequentially determined using an ELISA assay in patients with acute stroke, as compared to S100 and NSE. A diagnostic assay for stroke using FABP has been described in WO 01/42793. Use of the polypeptides according to the present invention can be validated in a similar way.

According to a first object of the invention, compositions are provided which comprise polypeptides for which the level was found increased in the cerebrospinal fluid from deceased patients compared to cerebrospinal fluid from healthy donors. According to this same object, compositions are disclosed which comprise antibodies which are derived from the above polypeptides

According to a second object of the invention, methods are provided which utilize the inventive compositions in the diagnosis and prognosis of brain damage-related disorders including cerebrovascular, dementia and neurodegenerative diseases.

The present invention provides the following:

1 A method of diagnosis of a brain damage-related disorder or the possibility thereof in a subject suspected of suffering therefrom, which comprises detecting at least one polypeptide, or a variant or mutant thereof, selected from A-FABP, E-FABP, H-FABP, B-FABP, PGP 9.5, GFAP, Prostaglandin D synthase, Neuromodulin, Neurofilament L, Calcyphosine, RNA binding regulatory subunit, Ubiquitin fusion degradation protein 1 homolog, Nucleoside diphosphate kinase A, Glutathione S tranferase P, Cathepsin D, DJ-1 protein, Peroxiredoxin 5 and Peptidyl-prolyl cis-trans isomerase A (Cyclophilin A) in a sample of body fluid taken from the subject.

2 A method according to 1, in which the polypeptide is differentially contained in the body fluid of brain damage-related disorder-affected subjects and non-brain damage-related disorder-affected subjects, and the method includes determining whether the concentration of polypeptide in the sample is consistent with a diagnosis of brain damage-related disorder.

3 A method according to 1 or 2, in which an antibody to the polypeptide is used in the detection or the determination of the concentration.

4 A method according to any of 1 to 3, in which the body fluid is cerebrospinal fluid, plasma, serum, blood, tears, urine or saliva.

5 A method according to any of 1 to 4, in which the polypeptide is present in the body fluid of brain damage-related disorder-affected subjects and not present in the body fluid of non-brain damage-related disorder-affected subjects, whereby the presence of the polypeptide in a body fluid sample is indicative of brain damage-related disorder.

6 A method according to any of 1 to 4, in which the polypeptide is not present in the body fluid of brain damage-related disorder-affected subjects and present in the body fluid of non-brain damage-related disorder-affected subjects, whereby the non-presence of the polypeptide in a body fluid sample is indicative of brain damage-related disorder.

7 A method according to any of 1 to 6, in which a plurality of peptides is determined in the sample.

8 A method according to any of 1 to 7, in which the polypeptide is differentially subject to post-translational modification in the body fluid of brain damage-related disorder-affected subjects and non-brain damage-related disorder-affected subjects, and the method includes detecting the post-translational modification of the polypeptide in the sample and determining whether this is consistent with a diagnosis of a brain damage-related disorder.

9 A method according to 8, in which the post-translational modification comprises N-glycosylation.

10 A method according to any of 1 to 9, in which the brain damage-related disorder is stroke and the polypeptide is Ubiquitin fusion degradation protein 1 homolog.

11 A method according to any of 1 to 9, in which the brain damage-related disorder is stroke and the polypeptide is RNA binding regulatory subunit.

12 A method according to any of 1 to 9, in which the brain damage-related disorder is stroke and the polypeptide is Nucleoside diphosphate kinase A.

13 A method according to any of 10 to 12, in which two or more markers selected from antibodies to Ubiquitin fusion degradation protein 1 homolog, RNA binding regulatory subunit, Nucleoside diphosphate kinase A and H-FABP are used in a single well of an ELISA microtiter plate.

14 A method according to 13, in which all four markers are used in a single well.

15 A method according to any of 10 to 12, in which two or more polypeptides selected from Ubiquitin fusion degradation protein 1 homolog, RNA binding

regulatory subunit, Nucleoside diphosphate kinase A and H-FABP are separately assayed, and a predictive algorithm is used for diagnosis.

16 Use of a polypeptide, or a variant or mutant thereof, selected from A-FABP, E-FABP, H-FABP, B-FABP, PGP 9.5, GFAP, Prostaglandin D synthase, Neuromodulin, Neurofilament L, Calcyphosine, RNA binding regulatory subunit, Ubiquitin fusion degradation protein 1 homolog, Nucleoside diphosphate kinase A, Glutathione S tranferase P, Cathepsin D, DJ-1 protein, Peroxiredoxin 5 and Peptidyl-prolyl cis-trans isomerase A (Cyclophilin A), or a combination of such polypeptides, for diagnostic, prognostic and therapeutic applications relating to brain damage-related disorders.

17 Use according to 16, in which the polypeptide is differentially contained in a body fluid of brain damage-related disorder-affected subjects and non-brain damage-related disorder-affected subjects.

18 Use for diagnostic, prognostic and therapeutic applications, relating to brain damage-related disorders, of a material which recognises, binds to or has affinity for a polypeptide, or a variant or mutant thereof, selected from A-FABP, E-FABP, H-FABP, B-FABP, PGP 9.5, GFAP, Prostaglandin D synthase, Neuromodulin, Neurofilament L, Calcyphosine, RNA binding regulatory subunit, Ubiquitin fusion degradation protein 1 homolog, Nucleoside diphosphate kinase A, Glutathione S tranferase P, Cathepsin D, DJ-1 protein, Peroxiredoxin 5 and Peptidyl-prolyl cis-trans isomerase A (Cyclophilin A).

19 Use according to 18 of a combination of materials, each of which respectively recognises, binds to or has affinity for a polypeptide, or a variant or mutant thereof, selected from A-FABP, E-FABP, H-FABP, B-FABP, PGP 9.5, GFAP, Prostaglandin D synthase, Neuromodulin, Neurofilament L, Calcyphosine, RNA binding regulatory subunit, Ubiquitin fusion degradation protein 1 homolog, Nucleoside diphosphate kinase A, Glutathione S tranferase P, Cathepsin D, DJ-1 protein, Peroxiredoxin 5 and Peptidyl-prolyl cis-trans isomerase A (Cyclophilin A).

20 Use according to 18 or 19, in which the or each material is an antibody or antibody chip.

21 Use according to 20, in which the material is an antibody to A-FABP.

22 Use according to 20, in which the material is an antibody to E-FABP.

23 Use according to 20, in which the material is an antibody to PGP 9.5.

24 Use according to 20, in which the material is an antibody to GFAP.

25 Use according to 20, in which the material is an antibody to Prostaglandin D synthase.

26 Use according to 20, in which the material is an antibody to Neuromodulin.

27 Use according to 20, in which the material is an antibody to Neurofilament L.

28 Use according to 20, in which the material is an antibody to Calcyphosine.

29 Use according to 20, in which the material is an antibody to RNA binding regulatory subunit.

30 Use according to 20, in which the material is an antibody to Ubiquitin fusion degradation protein 1 homolog.

31 Use according to 20, in which the material is an antibody to Nucleoside diphosphate kinase A.

32 Use according to 20, in which the material is an antibody to Glutathione S tranferase P.

33 Use according to 20, in which the material is an antibody to Cathepsin D.

34 Use according to 20, in which the material is an antibody to DJ-1 protein.

35 Use according to 20, in which the material is an antibody to Peroxiredoxin 5.

36 Use according to 20, in which the material is an antibody to Peptidyl-prolyl cis-trans isomerase A (Cyclophilin A).

37 An assay device for use in the diagnosis of brain damage-related disorders, which comprises a solid substrate having a location containing a material which recognizes, binds to or has affinity for a polypeptide, or a variant or mutant thereof, selected from A-FABP, E-FABP, H-FABP, B-FABP, PGP 9.5, GFAP, Prostaglandin D synthase, Neuromodulin, Neurofilament L, Calcyphosine, RNA binding regulatory subunit, Ubiquitin fusion degradation protein 1 homolog, Nucleoside diphosphate kinase A, Glutathione S tranferase P, Cathepsin D, DJ-1 protein, Peroxiredoxin 5 and Peptidyl-prolyl cis-trans isomerase A (Cyclophilin A).

38 An assay device according to 37, in which the solid substrate has a plurality of locations each respectively containing a material which recognizes, binds to or has affinity for a polypeptide, or a variant or mutant thereof, selected from A-FABP, E-FABP, H-FABP, B-FABP, PGP 9.5, GFAP, Prostaglandin D synthase, Neuromodulin, Neurofilament L, Calcyphosine, RNA binding regulatory subunit, Ubiquitin fusion degradation protein 1 homolog, Nucleoside diphosphate kinase A, Glutathione S tranferase P, Cathepsin D, DJ-1 protein, Peroxiredoxin 5 and Peptidyl-prolyl cis-trans isomerase A (Cyclophilin A).

39 An assay device according to 37 or 38, in which the material is an antibody or antibody chip.

40 An assay device according to 39, which has a unique addressable location for each antibody, thereby to permit an assay readout for each individual polypeptide or for any combination of polypeptides.

41 An assay device according to any of 37 to 40, including an antibody to A-FABP.

42 An assay device according to any of 37 to 40, including an antibody to E-FABP.

43 An assay device according to any of 37 to 40, including an antibody to PGP 9.5.

44 An assay device according to any of 37 to 40, including an antibody to GFAP.

45 An assay device according to any of 37 to 40, including an antibody to Prostaglandin D synthase.

46 An assay device according to any of 37 to 40, including an antibody to Neuromodulin.

47 An assay device according to any of 37 to 40, including an antibody to Neurofilament L.

48 An assay device according to any of 37 to 40, including an antibody to Calcyphosine.

49 An assay device according to any of 37 to 40, including an antibody to RNA binding regulatory subunit.

50 An assay device according to any of 37 to 40, including an antibody to Ubiquitin fusion degradation protein 1 homolog.

51 An assay device according to any of 37 to 40, including an antibody to Nucleoside diphosphate kinase A.

52 An assay device according to any of 37 to 40, including an antibody to Glutathione S tranferase P.

53 An assay device according to any of 37 to 40, including an antibody to Cathepsin D.

54 An assay device according to any of 37 to 40, including an antibody to DJ-1 protein.

55 An assay device according to any of 37 to 40, including an antibody to Peroxiredoxin 5.

56 An assay device according to any of 37 to 40, including an antibody to Peptidyl-prolyl cis-trans isomerase A (Cyclophilin A).

57 A kit for use in the diagnosis of brain damage-related disorders, comprising an assay device according to any of 37 to 56, and means for detecting the amount of one or more of the polypeptides in a sample of body fluid taken from a subject.

The new markers used in the present invention are as follows:

A-FABP (P15090), which has the sequence (SEQ ID NO.1):

1CDAFVGTWKLVSSENFDDYMKEVGVGFATRKVAGMAKPNMIISVNGDVITIKSESTFKNTEISFILG
QEFDEVTADDRKVKSTITLDGGVLVHVQKWDGKSTTIKRKREDDKLVVECVMKGVTSTRVYERA
131

E-FABP (Q01469), which has the sequence (SEQ ID NO.2):

1MATVQQLEGRWRLVDSKGFDEYMKELGVGIALRKMGAMAKPDCIITCDGKNLTIKTESTLKTTQF
SCTLGEKFEETTADGRKTQTVCNFTDGALVQHQEWDGKESTITRKLKDGKLVVECVMNNVTCTRIY
EKVE 135

PGP 9.5 (P09936), which has the sequence (SEQ ID NO.3):

1 MQLKPMEINP EMLNKVLSRL GVAGQWRFVD VLGLEEESLG SVPAPACALL LLFPLTAQHE 60
NFRKKQIEEL KGQEVSPKVY FMKQTIGNSC GTIGLIHAVA NNQDKLGFED GSVLKQFLSE 120
TEKMSPEDRA KCFEKNEAIQ AAHDAVAQEG QCRVDDKVNF HFILFNNVDG HLYELDGRMP 180
FPVNHGASSE DTLLKDAAKV CREFTEREQG EVRFSAVALC KAA 223

GFAP (P14136), which has the sequence (SEQ ID NO.4):

1 MERRRITSAA RRSYVSSGEM MVGGLAPGRR LGPGTRLSLA RMPPPLPTRV DFSLAGALNA 60
GFKETRASER AEMMELNDRF ASYIEKVRFL EQQNKALAAE LNQLRAKEPT KLADVYQAEL 120
RELRLRLDQL TANSARLEVE RDNLAQDLAT VRQKLQDETN LRLEAENNLA AYRQEADEAT 180
LARLDLERKI ESLEEEIRFL RKIHEEEVRE LQEQLARQQV HVELDVAKPD LTAALKEIRT 240
QYEAMASSNM HEAEEWYRSK FADLTDAAAR NAELLRQAKH EANDYRRQLQ SLTCDLESLR 300
GTNESLERQM REQEERHVRE AASYQEALAR LEEEGQSLKD EMARHLQEYQ DLLNVKLALD 360
IEIATYRKLL EGEENRITIP VQTFSNLQIR ETSLDTKSVS EGHLKRNIVV KTVEMRDGEV 420
IKESKQEHKD VM 432

Prostaglandin D synthase (P41222), which has the sequence (SEQ ID NO.5):

23 APEAQVSV QPNFQQDKFL GRWFSAGLAS NSSWLREKKA 60
ALSMCKSVVA PATDGGLNLT STFLRKNQCE TRTMLLQPAG SLGSYSYRSP HWGSTYSVSV 120
VETDYDQYAL LYSQGSKGPG EDFRMATLYS RTQTPRAELK EKFTAFCKAQ GFTEDTIVFL 180
PQTDKCMTEQ

Neuromodulin (P17677), which has the sequence (SEQ ID NO.6):

1 MLCCMRRTKQ VEKNDDDQKI EQDGIKPEDK AHKAATKIQA SFRGHITRKK LKGEKKDDVQ 60
AAEAEANKKD EAPVADGVEK KGEGTTTAEA APATGSKPDE PGKAGETPSE EKKGEGDAAT 120
EQAAPQAPAS SEEKAGSAET ESATKASTDN SPSSKAEDAP AKEEPKQADV PAAVTAAAAT 180
TPAAEDAAAK ATAQPPTETG ESSQAEENIE AVDETKPKES ARQDEGKEEE PEADQEHA 238

Neurofilament L (P07196), which has the sequence (SEQ ID NO.7):

1 SSFSYEPYYS TSYKRRYVET PRVHISVRSG YSTARSAYSS YSAPVSSSLS VRRSYSSSSG 60
SLMPSLENLD LSQVAAISND LKSIRTQEKA QLQDLNDRFA SFIERVHELE QQNKVLEAEL 120
LVLRQKHSEP SRFRALYEQE IRDLRLAAED ATTNEKQALR GEREEGLEET LRNLQARYEE 180
EVLSREDAEG RLMERRKGAD EAALARAELE KRIDSLMDEI SFLKKVHEEE IAELQAQIQY 240
AQISVEMDVT KPDLSAALKD IRAQYEKLAA KNMQNAEEWF KSRFTVLTES AAKNTDAVRA 300
AKDEVSESRR LLKAKTLEIE ACRGMNEALE KQLQELEDKQ NADISAMQDT INKLENELRT 360
TKSEMARYLK EYQDLLNVKM ALDIEIAAYR KLLEGEETRL SFTSVGSITS GYSQSSQVFG 420
RSAYGGLQTS SYLMSTRSFP SYYTSHVQEE QTEVEETIEA SKAEEAKDEP PSEGEAEEEE 480
KDKEEAEEEE AAEEEEAAKE ESEEAKEEEE GGEGEEGEET KEAEEEEKKV EGAGEEQAAK 540
KKD 543

Calcyphosine (Q13938), which has the sequence (SEQ ID NO.8):

1 MDAVDATMEK LRAQCLSRGA SGIQGLARFF RQLDRDGSRS LDADEFRQGL AKLGLVLDQA 60
EAEGVCRKWD RNGSGTLDLE EFLRALRPPM SQAREAVIAA AFAKLDRSGD GVVTVDDLRG 120
VYSGRAHPKV RSGEWTEDEV LRRFLDNFDS SEKDGQVTLA EFQDYYSGVS ASMNTDEEFV 180
AMMTSAWQL 189

RNA binding regulatory subunit (O14805), also referred to as RNA-BP, which has the sequence (SEQ ID NO.9):

1 MASKRALVIL AKGAEEMETV IPVDVMRRAG IKVTVAGLAG KDPVQCSRDV VICPDASLED 60
AKKEGPYDVV VLPGGNLGAQ NLSESAAVKE ILKEQENRKG LIAAICAGPT ALLAHEIGFG 120
SKVTTHPLAK DKMMNGGHYT YSENRVEKDG LILTSRGPGT SFEFALAIVE ALNGKEVAAQ 180
VKAPLVLKD 189

Ubiquitin fusion degradation protein 1 homolog (Q92890), also referred to as UFD1 or UFDP1, which has the sequence (SEQ ID NO.10):

1 MFSFNMFDHP IPRVFQNRFS TQYRCFSVSM LAGPNDRSDV EKGGKIIMPP SALDQLSRLN 60
ITYPMLFKLT NKNSDRMTHC GVLEFVADEG ICYLPHWMMQ NLLLEEDGLV QLETVNLQVA 120
TYSKSKFCYL PHWMMQNLLL EEGGLVQVES VNLQVATYSK FQPQSPDFLD ITNPKAVLEN 180
ALRNFACLTT GDVIAINYNE KIYELRVMET KPDKAVSIIE CDMNVDFDAP LGYKEPERQV 240
QHEESTEGEA DHSGYAGELG FRAFSGSGNR LDGKKKGVEP SPSPIKPGDI KRGIPNYEFK 300
LGKITFIRNS RPLVKKVEED EAGGRFVAFS GEGQSLRKKG RKP 343

Nucleoside diphosphate kinase A (P15531), also referred to as NDK A, which has the sequence (SEQ ID NO.11):

1 MANCERTFIA IKPDGVQRGL VGEIIKRFEQ KGFRLVGLKF MQASEDLLKE HYVDLKDRPF 60
FAGLVKYMHS GPVVAMVWEG LNVVKTGRVM LGETNPADSK PGTIRGDFCI QVGRNIIHGS 120
DSVESAEKEI GLWFHPEELV DYTSCAQNWI YE 152

Glutathione S tranferase P (P09211), which has the sequence (SEQ ID NO.12):

1 PPYTVVYFPV RGRCAALRML LADQGQSWKE EVVTVETWQE GSLKASCLYG QLPKFQDGDL 60
TLYQSNTILR HLGRTLGLYG KDQQEAALVD MVNDGVEDLR CKYISLIYTN YEAGKDDYVK 120
ALPGQLKPFE TLLSQNQGGK TFIVGDQISF ADYNLLDLLL IHEVLAPGCL DAFPLLSAYV 180
GRLSARPKLK AFLASPEYVN LPINGNGKQ 209

Cathepsin D (P07339), which has the sequence (SEQ ID NO.13):

65 GPIPEV LKNYMDAQYY GEIGIGTPPQ CFTVVFDTGS SNLWVPSIHC KLLDIACWIH 120
HKYNSDKSST YVKNGTSFDI HYGSGSLSGY LSQDTVSVPC QSASSASALG GVKVERQVFG 180
EATKQPGITF IAAKFDGILG MAYPRISVNN VLPVFDNLMQ QKLVDQNIFS FYLSRDPDAQ 240
PGGELMLGGT DSKYYKGSLS YLNVTRKAYW QVHLDQVEVA SGLTLCKEGC EAIVDTGTSL 300
MVGPVDEVRE LQKAIGAVPL IQGEYMIPCE KVSTLPAITL KLGGKGYKLS PEDYTLKVSQ 360
AGKTLCLSGF MGMDIPPPSG PLWILGDVFI GRYYTVFDRD NNRVGFAEAA RL 412

DJ-1 protein (Q99497), which has the sequence (SEQ ID NO.14):

1 MASKRALVIL AKGAEEMETV IPVDVMRRAG IKVTVAGLAG KDPVQCSRDV VICPDASLED 60
AKKEGPYDVV VLPGGNLGAQ NLSESAAVKE ILKEQENRKG LIAAICAGPT ALLAHEIGCG 120
SKVTTHPLAK DKMMNGGHYT YSENRVEKDG LILTSRGPGT SFEFALAIVE ALNGKEVAAQ 180
VKAPLVLKD 189

Peroxiredoxin 5 (P30044), which has the sequence (SEQ ID NO.15):

1 MGLAGVCALR RSAGYILVGG AGGQSAAAAA RRCSEGEWAS GGVRSFSRAA AAMAPIKVGD 60
AIPAVEVFEG EPGNKVNLAE LFKGKKGVLF GVPGAFTPGC SKTHLPGFVE QAEALKAKGV 120
QVVACLSVND AFVTGEWGRA HKAEGKVRLL ADPTGAFGKE TDLLLDDSLV SIFGNRRLKR 180FSMVVQDGIV KALNVEPDGT GLTCSLAPNI ISQL 214

Peptidyl-prolyl cis-trans isomerase A (Cyclophilin A) (P05092), which has the sequence (SEQ ID NO.16):

1 VNPTVFFDIA VDGEPLGRVS FELFADKVPK TAENFRALST GEKGFGYKGS CFHRIIPGFM 60
CQGGDFTRHN GTGGKSIYGE KFEDENFILK HTGPGILSMA NAGPNTNGSQ FFICTAKTEW 120
LDGKHVVFGK VKEGMNIVEA MERFGSRNGK TSKKITIADC GQLE 164

The polypeptides useful in the present invention are not restricted to the above sequences, and include variants and mutants thereof. A variant is defined as a naturally ocurring variation in the sequence of a polypeptide which has a high degree of homology with the given sequence, and which has substantially the same functional and immunological properties. A mutant is defined as an artificially created variant. A high degree of homology is defined as at least 90%, preferably at least 95% and most preferably at least 99% homology. Variants may occur within a single species or between different species. The above sequences are of human origin, but the invention encompasses use of the corresponding polypeptides from other mammalian species, e.g. bovine animals.

Brain damage-related disorders in the context of the present invention include the following : head trauma, ischemic stroke, hemorrhagic stroke, subarachnoid hemorrhage, intra cranial hemorrhage, transient ischemic attack, vascular dementia, corticobasal ganglionic degeneration, encephalitis, epilepsy, Landau-Kleffner syndrome, hydrocephalus, pseudotumor cerebri, thalamic diseases, meningitis, myelitis, movement disorders, essential tremor, spinal cord diseases, syringomyelia, Alzheimer's disease (early onset), Alzheimer's disease (late onset), multi-infarct dementia, Pick's disease, Huntingdon's disease, Parkinson, Parkinson syndromes, frontotemporal dementia, corticobasal degeneration, multiple system atrophy, progressive supranuclear palsy, Lewy body disease, amyotrophic lateral sclerosis, Creutzfeldt-Jakob disease, Dandy-Walker syndrome, Friedreich ataxia, Machado-Joseph disease, migraine, schizophrenia, mood disorders and depression. Corresponding disorders in non-human mammals

are also included, such as transmissible spongiform encephalopathies (TSEs), e.g. bovine spongiform encephalopathy (BSE) in cattle or scrapie in sheep.

H-FABP (P05413) and B-FABP (O15540) are also useful in the present invention for diagnosis of brain damage-related disorders or the possibility thereof, especially those other than stroke and CJD.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows results of an assay for H-FABP (measured in OD units on the vertical axis) for three groups of patients: a control group, a group with acute myocardial infarction (AMI), and a group with acute stroke;

Figure 2 shows the results of sequential determination of H-FABP levels (measured in OD units on the vertical axis) for the stroke group of patients at different time intervals after stroke;

Figure 3 shows portions of 2-DE maps for healthy and post-mortem CSF, with upward-directed arrows indicating spots corresponding to RNA binding regulatory subunit or DJ-1 protein. Enlargements of healthy CSF and deceased CSF 2-DE maps are shown. Forty five µg of protein was loaded on a IPG gel (pH 3.5-10 NL, 18cm). Second dimension was a vertical gradient slab gel (9-16%T). Gel was silver stained. The spots corresponding to the RNA binding regulatory subunit or to the DJ-1 protein are indicated by upward-directed (red) arrows;

Figure 4 shows portions of 2-DE maps for healthy and post-mortem CSF, with the right-hand arrows indicating spots corresponding to peroxiredoxin 5. Enlargements of healthy CSF and deceased CSF 2-DE maps are shown. Forty five µg of protein was loaded on a IPG gel (pH 3.5-10 NL, 18cm). Second dimension was a vertical gradient slab gel (9-16%T). Gel was silver stained. The spot corresponding to Peroxiredoxin 5 is indicated by the right-hand (red) arrows;

Figure 5 shows portions of 2-DE maps for healthy and post-mortem CSF, with the right-hand pair of arrows indicating spots corresponding to peptidyl-prolyl cis-

trans isomerase A (cyclophylin A). Enlargements of healthy CSF and deceased CSF 2-DE maps are shown. Forty five µg of protein was loaded on a IPG gel (pH 3.5-10 NL, 18cm). Second dimension was a vertical gradient slab gel (9-16%T). Gel was silver stained. The spots corresponding to Cyclophylin A are indicated by the right-hand pair of (red) arrows;

Figure 6 shows ELISA intensity values for marker polypeptides obtained in a survey of stroke patients;

Figure 7 shows UFD1 detection in plasma samples from said survey;

Figure 8 is an ROC curve of UFD1 from the data in Figure 7;

Figure 9 shows UFD1 detection corresponding to Figure 7;

Figure 10 shows RNA-BP detection in plasma samples from said survey;

Figure 11 is an ROC curve of RNA-BP from the data in Figure 10;

Figure 12 shows RNA-BP detection corresponding to Figure 10;

Figure 13 shows NDK A detection in plasma samples from said survey;

Figure 14 is an ROC curve of NDK A from the data in Figure 13;

Figure 15 shows NDK A detection corresponding to Figure 13;

Figure 16 shows portions of 2-DE maps for healthy and post-mortem CSF indicating prostaglandin D synthase levels;

Figure 17 shows prostaglandin D2 synthase spot intensities on mini-2-DE gels prepared with CSF of a CJD patient and a healthy patient as a control;

Figure 18 shows ELISA intensity values for H-FABP obtained in a survey of stroke patients and a control group;

Figure 19 shows UFDP-1 spot intensities on mini-2-DE-gels prepared with CSF from a control and a deceased patient;

Figure 20 shows UFDP1 plasma concentration measured by ELISA for two cohorts of stroke patients and controls from Geneva and from the USA;

Figure 21 shows RNA-BP spot intensities on mini-2-DE-gels prepared with CSF from a control and a deceased patient;

Figure 22 shows RNA-BP plasma concentration measured by ELISA for three studies of controls and stroke patients;

Figure 23 shows NDKA spot intensities on mini-2-DE-gels prepared with CSF from a control and a deceased patient;

Figure 24 shows NDKA plasma concentration measured by ELISA for two cohorts of stroke patients and controls from Geneva and from the USA;

Figure 25a shows the time onset of symptoms, showing the stroke marker (SM) concentration for UFDP1, RNA-BP and NDKA, in each case respectively for controls, stroke patients at less than 3 hours from the time of cerebrovascular accident, and stroke patients at more than 3 hours from the time of cerebrovascular accident;

Figure 25b shows data for type of stroke, showing the stroke marker concentration for UFDP1, RNA-BP and NDKA, in each case respectively for controls, hemorrhagic stroke patients, transient ischemic attack(TIA) patients and ischemic stroke patients;

Figure 26 is a summary of information for a panel of early plasmatic markers of stroke;

Figure 27 shows ELISA intensity values for a mix of UFD1, RNA-BP, NDKA and H-FABP in the same well;

Figure 28 is a graphic representation of combinations of two out of the four biomarkers from Figure 27, showing selected cut-off values for diagnosis;

Figures 29A and 29B show information related to 37 stroke and 37 age/sex matched control plasma samples in a further study. Diagnosis (Diag) is shown as I (ischemic stroke), H (hemorrhagic stroke), TIA (transient ischemic attack) or ctrl (control). The concentrations determined by ELISA of UFD1, RNA-BP and NDK A are also shown. ELISA was performed as previously described;

Figure 30 shows the results from this further study for 37 stroke and 37 control plasma samples tested in Geneva for UFD1. USA-1 (non age sex matched controls) data for UFD1;

Figure 31 shows the results from this further study for 37 stroke and 37 control plasma samples tested in Geneva for RNA-BP. USA-1 (non age sex matched controls) and USA-2 (age sex matched controls) data for RNA-BP;

Figure 32 shows the results of a large scale study USA3 on 633 patients for RNA-BP;

Figure 33 shows a statistical analysis (Kruskal-Wallis) on USA-3 for RNA-BP;

Figure 34 shows results for 33 stroke and 33 control plasma samples tested in Geneva for NDKA. USA-1 (non age sex matched controls) data for NDK A;

Figure 35 shows results of a large scale study USA3 on 622 patients for NDKA;

Figure 36 shows a statistical analysis (Kruskal-Wallis) on USA-3 for NDK A;

Figure 37 shows stroke marker concentration as a function of time onset of

symptoms (Geneva data, new 37 stroke and 37 control plasma samples);

Figure 38 shows stroke marker concentration as a function of type of stroke (hemorrhagic, ischemic, TIA) using USA-1 data.

DESCRIPTION OF PREFERRED EMBODIMENTS

The invention presented here is directed towards compositions and methods for detecting increasing or reducing polypeptides levels in body fluids including blood components (e.g. plasma or serum) or cerebrospinal fluid from patients affected by a brain damage-related disorder including cerebrovascular, dementia and neurodegenerative diseases. For this purpose, use can be made of antibodies or any specific polypeptide detection method.

Antibodies against brain damage protein markers, in particular their protein-binding domains, are suitable as detection tools. Molecular biological and biotechnological methods can be used to alter and optimize the antibody properties of the said molecules in a specific manner. In addition to this, the antibodies can be modified chemically, for example by means of acetylation, carbamoylation, formylation, biotinylation, acylation, or derivatization with polyethylene glycol or hydrophilic polymers, in order to increase their stability.

A specific polypeptide marker selected from A-FABP, E-FABP and any other FABP, i.e. H-FABP or B-FABP, PGP 9.5, GFAP, Prostaglandin D synthase, Neuromodulin, Neurofilament L, Calcyphosine, RNA binding regulatory subunit, Ubiquitin fusion degradation protein 1 homolog, Nucleoside diphosphate kinase A, Glutathione S tranferase P, Cathepsin D, DJ-1 protein, Peroxiredoxin 5 and Peptidyl-prolyl cis-trans isomerase A (Cyclophilin A) is determined in a body fluid sample, for example by using an antibody thereto. The marker is preferably measured by an immunoassay, using a specific antibody to the polypeptide and measuring the extent of the antigen (polypeptide)/antibody interaction. The antibody may be a monoclonal antibody or an engineered (chimeric) antibody. Antibodies to the polypeptides are known and are commercially available. Also, the usual Köhler-Milstein method may be used to raise antibodies. Less

preferably, the antibody may be polyclonal. In the context of the present invention, the term "antibodies" includes binding fragments of antibodies, such as single chain or Fab fragments.

Any known method of immunoassay may be used. In a sandwich assay an antibody (e.g. polyclonal) to the polypeptide is bound to the solid phase such as a well of a plastics microtitre plate, and incubated with the sample and with a labelled second antibody specific to the polypeptide to be detected. Alternatively, an antibody capture assay (also called "indirect immunoassay") can be used. Here, the test sample is allowed to bind to a solid phase, and the anti-polypeptide antibody (polyclonal or monoclonal) is then added and allowed to bind. If a polyclonal antibody is used in this context, it should desirably be one which exhibits a low cross-reactivity with other forms of polypeptide. After washing away unbound material, the amount of antibody bound to the solid phase is determined using a labelled second antibody, anti- to the first.

A direct assay can be performed by using a labelled anti-polypeptide antibody. The test sample is allowed to bind to the solid phase and the anti-polypeptide antibody is added. After washing away unbound material, the amount of antibody bound to the solid phase is determined. The antibody can be labelled directly rather than via a second antibody.

In another embodiment, a competition assay can be performed between the sample and a labelled polypeptide or a peptide derived therefrom, these two antigens being in competition for a limited amount of anti-polypeptide antibody bound to a solid support. The labelled polypeptide or peptide can be pre-incubated with the antibody on the solid phase, whereby the polypeptide in the sample displaces part of the polypeptide or peptide thereof bound to the antibody.

In yet another embodiment, the two antigens are allowed to compete in a single co-incubation with the antibody. After removal of unbound antigen from the support by washing, the amount of label attached to the support is determined and the amount of protein in the sample is measured by reference to standard titration curves established previously.

Throughout, the label is preferably an enzyme. The substrate for the enzyme may be colour-forming, fluorescent, chemiluminescent or electrochemical, and can be soluble or precipitating. Alternatively, the label may be a radioisotope or fluorescent, e.g. using conjugated fluorescein.

The enzyme may, for example, be alkaline phosphatase or horseradish peroxidase and can conveniently be used colorimetrically, e.g. using p-nitrophenyl phosphate as a yellow-forming substrate with alkaline phosphatase.

For a chemiluminescent assay, the antibody can be labelled with an acridinium ester or horseradish peroxidase. The latter is used in enhanced chemiluminescent (ECL) assay. Here, the antibody, labelled with horseradish peroxidase, participates in a chemiluminescent reaction with luminol, a peroxide substrate and a compound, which enhances the intensity and duration of the emitted light, typically, 4-iodophenol or 4-hydroxycinnamic acid.

An amplified immunoassay such as immuno-PCR can be used. In this technique, the antibody is covalently linked to a molecule of arbitrary DNA comprising PCR primers, whereby the DNA with the antibody attached to it is amplified by the polymerase chain reaction. See E. R. Hendrickson *et al.*, Nucleic Acids Research 1995; 23, 522-529 (1995) or T. Sano et al., in "Molecular Biology and Biotechnology" ed. Robert A. Meyers, VCH Publishers, Inc. (1995), pages 458 - 460. The signal is read out as before.

In one procedure, an enzyme-linked immunosorbent assay (ELISA) can be used to detect the polypeptide.

The use of a rapid microparticle-enhanced turbidimetric immunoassay, developed for H-FABP in the case of AMI, M.Robers *et al.*, "Development of a rapid microparticle-enhanced turbidimetric immunoassay for plasma fatty acid-binding protein, an early marker of acute myocardial infarction", Clin. Chem. 1998;44:1564-1567, significantly decreases the time of the assay. Thus, the full automation in a widely used clinical chemistry analyser such as the COBAS™

MIRA Plus system from Hoffmann-La Roche, described by M.Robers *et al. supra*, or the AxSYM™ system from Abbott Laboratories, should be possible and applied for routine clinical diagnosis of brain damage-related disorders.

The polypeptide concentrations can be measured by other means than immunoassay. For example, the sample can be subjected to 2D-gel electrophoresis and the amount of the polypeptide estimated by densitometric scanning of the gel or of a blot therefrom. However, it is desirable to carry out the assay in a rapid manner, so that the patient can be treated promptly.

In principle, any body fluid can be used to provide a sample for diagnosis, but preferably the body fluid is cerebrospinal fluid (CSF), plasma, serum, blood, urine, tears or saliva.

According to the invention, a diagnosis of brain damage-related disorders may be made from determination of a single polypeptide or any combination of two or more of the polypeptides.

The invention also relates to the use of one or more of the specified polypeptides which is differentially contained in a body fluid of brain damage-affected subjects and non-brain damage-affected subjects, for diagnostic, prognostic and therapeutic applications. This may involve the preparation and/or use of a material which recognizes, binds to or has some affinity to the above-mentioned polypeptide. Examples of such materials are antibodies and antibody chips. The term "antibody" as used herein includes polyclonal antiserum, monoclonal antibodies, fragments of antibodies such as Fab, and genetically engineered antibodies. The antibodies may be chimeric or of a single species. The above reference to "prognostic" applications includes making a determination of the likely course of a brain damage-related disorder by, for example, measuring the amount of the above-mentioned polypeptide in a sample of body fluid. The above reference to "therapeutic follow-up" applications includes making a determination of the likely course of a brain damage-related disorder by, for example, measuring the amount of the above-mentioned polypeptide in a sample of body fluid (and evaluating its level as a function of the treatment, the disability recovery or not, the size of the

lesions etc.). The above reference to "therapeutic" applications includes, for example, preparing materials which recognize, bind to or have affinity to the above-mentioned polypeptides, and using such materials in therapy. The materials may in this case be modified, for example by combining an antibody with a drug, thereby to target the drug to a specific region of the patient.

The above reference to "presence or absence" of a polypeptide should be understood to mean simply that there is a significant difference in the amount of a polypeptide which is detected in the affected and non-affected sample. Thus, the "absence" of a polypeptide in a test sample may include the possibility that the polypeptide is actually present, but in a significantly lower amount than in a comparative test sample. According to the invention, a diagnosis can be made on the basis of the presence or absence of a polypeptide, and this includes the presence of a polypeptide in a significantly lower or significantly higher amount with reference to a comparative test sample.

The above references to "detecting" a polypeptide should be understood to include a reference to compositions and methods for detecting post-translational modifications of the polypeptides in addition to quantitative variations. As an example, we detected differences in the post-translational modifications pattern of prostaglandin D synthase between post-mortem and control CSF. Similar differences were also detected between CSF from a patient suffering from Creutzfeldt-Jakob disease and control CSF. This is described in Example 5 below. The invention therefore encompasses the detection of post-translational modifications in general, and determining whether such modifications of a polypeptide are consistent with a diagnosis of a brain damage-related disorder.

Kits and assay devices for use in diagnosis of brain damage-related disorders are also within the scope of the invention. These may include one or more antibodies to a polypeptide selected from A-FABP, E-FABP and any other FABP, i.e. H-FABP or B-FABP, PGP 9.5, GFAP, Prostaglandin D synthase, Neuromodulin, Neurofilament L, Calcyphosine, RNA binding regulatory subunit, Ubiquitin fusion degradation protein 1 homolog, Nucleoside diphosphate kinase A, Glutathione S tranferase P, Cathepsin D, DJ-1 protein, Peroxiredoxin 5 and Peptidyl-prolyl cis-

trans isomerase A (Cyclophilin A). The antibodies will bind to the appropriate polypeptides in a fluid sample taken from a patient. The antibodies may be immobilised on a solid support. Preferably, each antibody is placed in a unique addressable location, thereby to permit separated assay readout for each individual polypeptide in the sample, as well as readouts for any selected combination of polypeptides.

The following Examples illustrate the invention.

EXAMPLE 1

Using two-dimensional gel electrophoresis (2-DE) separation of cerebrospinal fluid (CSF) proteins and mass spectrometry techniques, 15 polypeptides named in Table 1 were found elevated or decreased in the CSF of deceased patients, used as a model of massive brain damage.

Study population and samples handling

Eight CSF samples were used for the proteomics-based approach aiming at discovering brain damage-related disorder markers. Four of these samples were obtained at autopsy from deceased patients 6 hours after death with no pathology of the central nervous system. Four others were collected by lumbar puncture from living patients who had a neurological workup for benign conditions unrelated to brain damage (atypical headache and idiopathic peripheral facial nerve palsy). CSF samples were centrifuged immediately after collection, aliquoted, frozen at -80°C and stored until analysis.

CSF 2-DE

All reagents and apparatus used have been described in detail elsewhere [9]. 250µl of CSF were mixed with 500µl of ice-cold acetone (-20°C) and centrifuged at 10000g at 4°C for 10 minutes. The pellet was mixed with 10µl of a solution containing 10% SDS (w/v) and 2.3% DTE (w/v). The sample was heated to 95°C for 5 minutes and then diluted to 60µl with a solution containing 8M urea, 4% CHAPS (w/v), 40mM Tris, 65mM DTE and a trace of bromophenol blue. The whole final diluted CSF sample corresponding to 45µg was loaded in a cup at the

cathodic end of the IPG strips. 2-DE was performed as described previously [10]. In brief, the first dimensional protein separation was performed using a commercial 18cm non-linear IPG going from pH 3.5 to 10 from Amersham Biosciences (Uppsala, Sweden). The second dimensional separation was performed onto in-house manufactured vertical gradient slab gels (9-16% T, 2.6% C). Analytical gels were then stained with ammoniacal silver staining [11]. Gels were scanned using a laser densitometer (Amersham Biosciences, Uppsala, Sweden). 2-DE computer image analysis was carried out with the MELANIE 3 software package [12].

Mass spectrometry identification

Differentially expressed spots were found through the comparison of analytical gels of deceased vs. healthy CSF (n=4). Spots of interest were analysed by peptide mass fingerprinting using a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (PerSeptive Biosystems Voyager STR MALDI-TOF-MS, Framingham, MA, USA) [10] and identified through database using the PeptIdent tool (http://www.expasy.ch/sprot/peptident.html).

Table 1:

A-FABP	P15090
E-FABP	Q01469
PGP 9.5	P09936
GFAP	P14136
Prostaglandin D synthase	P41222
Neuromodulin	P17677
Neurofilament L	P07196
Calcyphosine	Q13938
RNA binding regulatory subunit	O14805
Ubiquitin fusion degradation protein 1 homolog	Q92890
Nucleoside diphosphate kinase A	P15531
Glutathione S tranferase P	P09211
Cathepsin D	P07339
H-FABP	P05413
B-FABP	O15540

EXAMPLE 2

Using two-dimensional gel electrophoresis (2-DE) separation of cerebrospinal fluid (CSF) proteins and mass spectrometry techniques, FABP was found elevated in the CSF of deceased patients, used as a model of massive brain damage. Since H-FABP, a FABP form present in many organs, is also localised in the brain, an enzyme-linked immunosorbant assay (ELISA) was developed to detect H-FABP in stroke vs. control plasma samples. However, H-FABP being also a marker of acute myocardial infarction (AMI), Troponin-I and creatine kinase-MB (CK-MB) levels were assayed at the same time in order to exclude any concomitant heart damage. NSE and S100B levels were assayed simultaneously.

Study population and samples handling

The population used for the assessment in plasma of the various markers detailed below included a total of 64 prospectively studied patients (Table 2) equally distributed into three groups: (1) a Control group including 14 men and 8 women aged 65 years (ranges: 34-86 years) with no known peripheral or central nervous system condition; (2) a group of patients with acute myocardial infarction (AMI group) including 14 men and 6 women aged 65 years (ranges: 29 to 90 years); the diagnosis of AMI was established in all cases by typical electrocardiography modifications and elevated levels of CK-MB (above a cut-off value of 9.3 ng/ml) and of Troponin-I (above a cut-off value of 2 ng/ml) ; (3) a group of patients with acute stroke (Stroke group) including 14 men and 8 women aged 65 years (ranges: 30 to 87 years); the diagnosis of stroke was established by a trained neurologist and was based on the sudden appearance of a focal neurological deficit and the subsequent delineation of a lesion consistent with the symptoms on brain CT or MRI images, with the exception of transient ischemic attacks (TIAs) where a visible lesion was not required for the diagnosis. The Stroke group was separated according to the type of stroke (ischemia or haemorrhage), the location of the lesion (brainstem or hemisphere) and the clinical evolution over time (TIA when complete recovery occurred within 24 hours, or established stroke when the neurological deficit was still present after 24 hours).

Table 2

Group	Control	AMI	Stroke	Stroke					
				Diagnosis		Location		Type	
				Ischemia	Haemorrhage	Brainstem	Hemisphere	TIA	CVA
Diagnosis Number Stroke	22	20	22 22						
H-FABP									
OD>0.531	0	20	15	11	4	3	12	3	12
OD<0.531	22	0	7	5	2	1	6	2	5
Troponin-I									
>2ng/ml	0	20	1						
<2ng/ml	22	0	21						

For each patient of the three groups, a blood sample was collected at the time of admission in dry heparin-containing tubes. After centrifugation at 1500g for 15min at 4°C, plasma samples were aliquoted and stored at –20°C until analysis. For the Stroke group, three additional blood samples were collected after the neurological event: <24 hours; <48 hours; and >48 hours. In this group, the time interval between the neurological event and the first blood draw was 185 minutes (ranging from 40 minutes to 3 days). This parameter was taken into account in the data analysis. Each patient or patient's relatives gave informed consent prior to enrolment.

FABP measurement

H-FABP levels were measured in plasma by a sandwich ELISA. A 96-well polystyrene microtitre plate (NUNC, Polylabo, CH) was coated with 100µl/well polyclonal goat anti human muscle FABP (Spectral Diagnosis HC, Ontario, USA), 20.4µg/ml in carbonate buffer 0.1M pH 9.6, overnight at 4°C. The plate was automatically washed with PBS (15mM Na_2PO_4-120mM NaCl-2.7mM KCl pH 7.4, Sigma) on a BioRad NOVAPATH™ WASHER (Hercules, CA). Every washing step was performed with fresh PBS. Non-specific binding sites were blocked with 200µl/well 2% casein (w/v) in carbonate buffer for 2h at 37°C. After the washing step, the samples were pipetted in duplicate at 100µl/well. The plate was incubated 2h at 37°C. After the washing step, 100µl/well of mouse anti-human Heart FABP (clone 66E2, HyCult biotechnology b.v, Uden,

Netherlands), 0.3µg/ml in PBS-1% BSA (w/v), were incubated for 1h at room temperature (R.T) with shaking. After the washing step, 100µl/well of phosphatase labelled anti-mouse immunoglobulins (Dako, Denmark), 15µg/ml in PBS, were incubated 1h30 at R.T. with shaking. After the washing step, 50µl/well of phosphatase substrate, 1.5mg/ml paranitrophenylphosphate in diethanolamine, were incubated 30min. Reaction was stopped with 100µl/well NaOH 1M. Colour development was assessed with a microplate reader, *Milenia*™ kinetic analyzer (DPC, LA, USA), at a wavelength of 405nm.

CK-MB and Troponin-I measurement

Plasma samples were centrifuged at 1500g for 15min, and aliquots were stored at –20°C. Serum CK-MB and Troponin-I levels were determined using a fluorescent microparticle enzyme immunoassay (MEIA) with an automated chemical analyser AxSYM™ system (ABBOTT Laboratories, Abbott Park, IL). The formation rate of fluorescent products was directly proportional to the amount of Troponin-I in the sample. The detection limit for Troponin-I was 0.3µg/l. CK-MB measurement is proportional to the amount of fluorescent probes and the detection limit was 0.7µg/l.

NSE and S100 measurement

Similar to H-FABP measurements, NSE and S100B were assayed in the four serial plasma samples of the Stroke group. The SMART S-100 and SMART-NSE ELISA kits were used. Both have been commercialised by Skye PharmaTech Inc. (Ontario, CA). The detection limits for NSE and S100B were 1µg/l and 0.01µg/l respectively.

Statistical analysis

H-FABP levels were expressed in optical density (OD) values as mean $\pm$ SD. Because recombinant H-FABP was not available, external calibration could not be performed to express results as concentration units (ng/ml). Troponin-I and CK-MB levels, were expressed in ng/ml. Because plasma H-FABP, troponin-I and CK-MB concentrations did not fulfill the criteria for a gaussian distribution in neither of the normal, stroke and AMI populations according to the Kolmogorov-Smirnov test, comparisons between the three groups was carried out using the

non-parametric Kruskall-Wallis test with post hoc Dunn's procedure. Comparisons between the stroke subgroups defined above were made by means of the Mann-Whitney *U* test and longitudinal assessment of H-FABP concentrations over time were analyzed using the repeated measures analysis of variance (ANOVA). Reference limits for H-FABP aiming at distinguishing stroke versus normal patients were delineated using receiver operating characteristic (ROC) curves (Analyse-It™ software for Microsoft Excel™) and, subsequently, sensitivity, specificity, positive and negative predictive values were calculated at each time point. Statistical significance was set at $p<0.05$.

Results

Individual results of the H-FABP assay in the three populations, expressed in OD units, are graphically shown in Figure 1 and detailed in Table 3. Mean plasma H-FABP concentration was 0.221 + 0.134 OD in the Control group, 1.079 ± 0.838 OD in the Stroke group and 2.340 ± 0.763 OD in the AMI group. The coefficient of variation found for this ELISA was $5.8\% \pm 3.8$. Using the Kruskall-Wallis test, all three concentrations were found significantly different ($p<0.001$) from each other. The best cut-off value to discriminate between the Control and the Stroke groups was set at OD > 0.531 as determined by the ROC curves for H-FABP level (data not shown). Using this cut-off value, validity measures of H-FABP for the diagnosis of stroke were as follows: sensitivity was 68.2 % with 15 out of 22 stroke patients above the cut-off, specificity was 100 % with all of the 22 control subjects below the cut-off, positive predictive value was 100 % and negative predictive value was 75.9 %.

Table 3

Group		Control	AMI	Stroke
H-FABP	mean	0.221	2.434	1.079
	SD	0.134	0.638	0.838
	Significance		<0.001	<0.001
Troponin-I	mean	0.0	164.6	0.5
	SD	0.1	205.6	1.3
	Significance		<0.001	ns
CK-MB	mean	1.3	63.8	7.9
	SD	0.9	51.5	21.3
	Significance		<0.001	ns

To discriminate, at the biological level, between patients from the AMI and the Stroke groups, Troponin-I and CK-MB were further assayed in each group with cut-off values set at 2 ng/ml for the AxSYM Troponin-I assay and 3.8 ng/ml for the AxSYM CK-MB assay (Table 3). As expected, the concentrations of these AMI markers were significantly higher ($p<0.01$) in the AMI group as compared to both the Control and the Stroke groups. No difference was found between the last two groups, thus confirming that Troponin-I and CK-MB do not increase as a result of a brain insult and that stroke patients did not sustain a concomitant AMI at the time of their stroke. Taken together, H-FABP, Troponin-I and CK-MB concentrations allowed a correct discrimination between AMI (increase of all three markers) and stroke (increase of H-FABP with normal Troponin-I and CK-MB) in all the 20 AMI patients and in 15 stroke patients, with the exception of one stroke patient showing, along with increased H-FABP levels, moderately elevated levels of Troponin-I and CK-MB in the absence of EKG modifications, all of which being consistent with a concomitant non-AMI heart damage.

In the Stroke group, seven false negative results were found with H-FABP levels below the cut-off value of OD 0.531 at any time point following the neurological event. Of these seven patients, two had a rapid and complete recovery of their neurological deficits within 24 hours consistent with a transient ischemic attack (TIA), and two have had a lacunar stroke on MRI images, one located in the brainstem. While TIA and lacunar stroke may explain false negative results in a majority of patients, no explanation was consistently found for the three remaining stroke patients with low H-FABP levels.

Sequential determinations of H-FABP level after stroke showed that 10 out of 15 (67 %) H-FABP positive stroke patients had a very early increase of H-FABP levels (<12 hours). Moreover, as shown in Figure 2, when all stroke patients were considered, the mean H-FABP concentrations decreased steadily after the insult, the highest value being found before 12 hours. The differences between the initial measurement and the less than 48 hours and afterwards measurements were significant (ANOVA, $p<0.05$). When H-FABP levels were compared between the different subgroups of the Stroke group, no statistically significant differences were found. H-FABP levels were similar for ischemia (0.955 ± 0.668, N=15)

versus haemorrhage (1.346 ± 1.139, N=7) strokes, and for hemispheric (0.987 ± 0.783, N=18) versus brainstem (1.493 ± 1.080) strokes, but the statistical power of the analyses was limited by the small size of the samples to be compared. However, when comparing established strokes versus TIAs, the former (1.2002 ± 0.892) showed nearly twice as high H-FABP levels as the latter (0.652 ± 0.499), although this difference failed to reach significance (Mann-Whitney *U* test, p=0.24).

Finally, NSE and S100B were assayed in the Control and the Stroke groups, and the results were compared with the H-FABP assay. The cut-off values using the SMART-NSE and SMART S100B protein ELISA tests for the diagnosis of stroke were 10 ng/ml for NSE and 0.02 ng/ml for S100B. NSE and S100B levels were slightly increased in the Stroke groups (14.12 ng/ml and 0.010 ng/ml, respectively) as compared to the Control group (15.88 ng/ml and 0.004 ng/ml, respectively). As shown on Table 4, specificity, sensitivity, PPV and NPV for the diagnosis of stroke were found much lower for NSE and S100B than for H-FABP. These differences are relevant since the three markers have been tested in the same samples.

Table 4

	H-FABP	NSE	S100B
Sensitivity	68.2	55	15
Specificity	100	36.4	95.5
Positive predictive	100	44	75
Negative predictive	75.9	47.1	55.3

EXAMPLE 3

Three new proteins have been identified on 2-DE gels prepared with CSF samples from deceased patients. These proteins correspond to spots that have been previously shown increased in CSF samples from deceased patients relative to healthy controls. However, previous attempts to identify these proteins using MALDI-TOF mass spectrometry were unsuccessful. The current experiments

were performed by μLC-MS-MS using ESI-Ion Trap device (DecaLCQ XP, ThermoFinnigan). Furthermore, the increasing amount of data in databases could lead to the successful identification of previously uncharacterized spots.

(1) RNA-binding protein regulatory subunit (O14805) / DJ-1 protein (Q99497):

RNA-binding protein regulatory subunit has been previously described in deceased CSF samples (see Example 1 above). Here, we have obtained the same identification with an adjacent spot (Figure 3). We also confirmed the previous identifications. Figure 1 shows enlargements of healthy CSF and deceased CSF 2-DE maps. 270 μg of protein was loaded on a IPG gel (pH 3.5 – 10NL, 18cm). The second dimension was a vertical slab gel (12 %T). The gel was stained with a fluorescent dye. The upward-pointing arrows indicate the spots corresponding to the RNA binding regulatory subunit or to the DJ-1 protein.

Furthermore, our results indicate that these spots could correspond to another homologous protein called DJ-1. The RNA-binding protein regulatory subunit and DJ-1 sequences differ from one another only by one amino acid. The single peptide detected during the current experiments did not contain this specific residue.

DJ-1 gene mutations are associated with autosomal recessive early-onset parkinsonism (Bonifati et al., Science, 2003). Different results suggest that the DJ-1 protein could be involved in cellular oxidative stress response and neurodegenerative pathologies (Bonifati et al., Science, 2003; Wilson et al., PNAS, 2003).

(2) Peroxiredoxin 5 (P30044):

The 2-DE spot corresponding to Peroxiredoxin 5 is shown in Figure 4. This is an enlargement of healthy CSF and deceased CSF 2-DE maps prepared in the same way as for Figure 3. The spot corresponding to Peroxiredoxin 5 is shown by the arrows on the right-hand side.

Peroxiredoxin 5 is an antioxidant enzyme that could have a neuroprotective effect (Plaisant et al., Free Radic. Biol. Med., 2003). Aberrant expression pattern of proteins belonging to the Peroxiredoxin family was also described in brains of patients with different neurodegenerative diseases (Krapfenbauer et al., Electrophoresis, 2002; Krapfenbauer et al., Brain Res., 2003).

(3) Peptidyl-prolyl cis-trans isomerase A or Cyclophilin A (P05092)

Two spots were identified as being the Peptidyl-prolyl cis-trans isomerase A (Figure 5). This is an enlargement of healthy CSF and deceased CSF 2-DE maps prepared in the same way as for Figure 4. The basic spot corresponding to Cyclophilin A is just adjacent to the spot corresponding to the Peroxiredoxin 5.

Cyclophilin A was described as a protective factor against cellular oxidative stress (Doyle et al., Biochem J., 1999). It binds to Peroxiredoxin enzymes and could be involved in the peroxidase activity (Lee et al., J.Biol. Chem., 2001). Furthermore, a publication suggests that Cyclophilin A is secreted by vascular smooth muscle cells (VSMC) in response to oxidative stress and stimulate VSMC growth (Jin et al., Circ. Res., 2000). These results suggest the implication of Cyclophilin A in vascular diseases processes. A link was also described with a familial form of amyotrophic lateral sclerosis (a neurodegenerative pathology) associated with a mutation in the antioxidant enzyme Cu/Zn Superoxide Dismutase-1 (Lee at al., PNAS, 1999). Cyclophilin A seems to have a protective effect against the mutant SOD-induced apoptosis.

EXAMPLE 4

Introduction

A survey of stroke patients was carried out and the results are shown in Figures 6 to 15. An ELISA intensity signal was obtained for Ubiquitin fusion degradation protein 1 homolog (UFD1), RNA binding regulatory subunit (RNA-BP) and nucleotide diphosphate kinase A (NDK A) in plasma samples of the patients and of negative control patients. Plasma samples were taken from patients between 0-

24 hours and/or after 72 hours of arrival at emergency hospital, and were matched for age/sex with samples from control patients.

Protocol

ELISA was performed using 96-well Reacti-Bind™ NeutrAvidin™ coated Black Plates (Pierce, Rockford, IL). Plates were first rinsed in Borate Buffer Saline pH 8.4 (BBS) (100 mM H_3BO_3, 25 mM $Na_2B_4O_7$ (Sigma, St Louis, MO, USA), 75 mM NaCl (Merck, Darmastadt, Germany)) on a NOVAPATH™ washer (Bio-Rad, Hercules, CA). Then, 50µl of antibody-biotin conjugated (2 µg/mL) prepared in the dilution buffer A at pH 7 (DB, Polyvinyl Alcohol, 80% hydrolyzed, Mol. Wt. 9000-10,000 (Aldrich, Milwaukee, WI, USA), MOPS (3-[N-Morpholino] propane sulfonic acid) (Sigma), NaCl, $MgCl_2$ (Sigma), $ZnCl_2$ (Aldrich), pH6.90, BSA 30% Solution, Manufacturing Grade (Serological Proteins Inc., Kankakee, IL)), were added and incubated for one hour at 37°C. Plates were then washed 3 times in BBS in the plate washer. 50 µl of antigen was then added and incubated for one hour at 37°C. Recombinant proteins were diluted at 100, 50, 25, 12.5, 6.25 ng/ml in the dilution buffer A to establish a calibration curve. Plasma samples were diluted at the appropriate dilution in the dilution buffer A. After the washing step, 50µl of alkaline phosphatase conjugated antibodies were added at the appropriate dilution in the dilution buffer A and incubated for one hour at 37°C. The 96-well plate was then washed 3 times with BBS in the plate washer and 50 µL of fluorescence Attophos® AP Fluorescent substrate (Promega, Madison, WI) were added. Plates were read immediately on a SpectraMax GEMINI-XS, (Molecular Devices Corporation, Sunnyvale, CA, U.S.A.) fluorometer microtiter plate reader relative fluorescence units (RFU) ($\lambda_{excitation}$=444 nm and $\lambda_{emission}$=555 nm).

We read plates in fluorescence using a SpectraMax GEMINI-XS (Molecular Devices) fluorometer microplate reader (λexcitation=444 nm and λemission=555 nm). Results are expressed in RFU and can be obtained in endpoint mode (only one reading) or in kinetic mode on 10 minutes. In kinetic mode, for each well we used 6 flashes (per well) which are integrated into an average and read each well 6 times using minimal interval time between each reading. This ends up being 2

minutes between readings. We determined a slope and this is what we used for our valuations. The best cut-off value to discriminate between the Control and the Stroke (Ischemic plus hemorrhagic or Ischemic vs. Hemorrhagic) groups was determined by the ROC curves using GraphPad Prism 4 software.

Conclusion

We can clearly see from Figures 7, 10 and 13 that UFD1, RNA-BP and NDK A respectively are overexpressed in stroke patients compared to control patients. Statistical analysis for each of the biomarker was performed and ROC curves (GraphPad Prism 4 software) indicating sensitivity of the test as a function of 1-specificity (Figures 8, 11 and 14 for UFD1, RNA-BP and NDK A respectively) were drawn. Best cutoff values to distinguish between stroke and control patients were deduced from these ROC curves. We obtained 94.4%, 94.4% and 100% sensitivity for UFD1, RNA-BP and NDK A respectively and 77.8%, 72.2% and 83.3% specificity for UFD1, RNA-BP and NDK A respectively. For each marker, a non parametric Mann Whitney test was performed to compare stroke and control groups. For the 3 biomarkers, we obtained very low p values (<0.0001 for UFD1 and NDK A and p=0.0003 for RNA-BP) meaning that differences between stroke and controls are very significant.

In Figure 6, we can also notice that RNA-BP and NDK A can detect a stroke only 30 minutes after symptoms onset, meaning that these are very early markers of stroke. This result is confirmed by the decreasing signal observed between arrival at the hospital and after 72 hours. Patients 202 and 239 were tested at the arrival (between 0 and 24 hours) and after 72 hours and we can see that for all the markers, the signal significantly decreases.

These results demonstrate that Ubiquitin fusion degradation protein 1 homolog (UFD1), RNA binding regulatory subunit (RNA-BP) and nucleotide diphosphate kinase A (NDK A) are useful markers for early diagnosis of stroke, alone, in combination, or combined with other biomarkers.

EXAMPLE 5

This Example is concerned with post-translational modifications that can be induced in neurodegenerative disorders. The study population and samples handling, and the CSF 2-DE were as described in Example 1.

2-DE immunoblotting assays

Proteins separated by 2-DE were electroblotted onto PVDF membranes essentially as described by Towbin *et al.* [22]. Membranes were stained with Amido Black, destained with water and dried. Proteins of interest were detected as previously described [29] using specific antibodies and ECL™ western blotting detection reagents (Amersham Biosciences, Uppsala, Sweden). We used the following antibody: anti-human Prostaglandin D synthase (lipocalin type) rabbit polyclonal antibody (Cayman chemical, Ann Arbor, MI) diluted 1/250.

Figure 16 (A) shows a comparison of PGHD spot intensities on 2-DE gels prepared with CSF of deceased or control patients. Forty-five µg of protein was loaded on an IPG strip (pH 3.5-10 NL, 18 cm). The second dimension was performed on a vertical gradient slab gel (9-16 % T), stained with ammoniacal silver. Apolipoprotein AI labelled in italic showed similar levels in the two samples. PGHD spot locations in control gel were deduced from previous identifications [31]. In the gel from deceased patients, putative PGHD spot locations are given. Figure 16(B) shows immunodetection of PGHD in 2-DE gels prepared with CSF from deceased and control patients. 2-DE was performed as indicated in A. Immunodetection was performed as previously described [29] using an anti-human Prostaglandin D synthase (lipocalin type) rabbit polyclonal antibody and ECL™ western blotting detection reagents.

Results

Prostaglandin D synthase (PGHD) is a basic protein (pI = 8.37) known to be post-translationaly modified by N-glycosylation (Hoffmann A. *et al.*, *J. Neurochem.* 1994, *63*, 2185-2196). On CSF 2-D gels from healthy living patients, five spots were detected. On 2-D gels prepared with post-mortem CSF, the three acidic

spots are strongly decreased with a concomitant increase of the two basic spots (Figure 16A).

In order to confirm that these different spots correspond to PGHD, we performed immunoblot assays using a specific antibody (Figure 16B). The results obtained confirmed that the acidic PGHD spots were not present in the CSF from deceased patients while the basic spots were still present. Furthermore, the measurement of the total PGHD spot volume in the two gels using the Melanie 3 software indicated that the PGHD level is similar in the two samples. This suggests, therefore, that there was a deglycosylation of PGHD in the CSF of deceased patients but the total PGHD amount remained constant.

Data from the literature:

PGHD was found to be decreased in the CSF of patients suffering from AD (Puchades M. *et al.*, *Brain Res. Mol. Brain Res.* 2003, *118*, 140-146). However, the study was performed using 2-DE gels and only the acidic spots were analyzed. As shown by our results on CSF from deceased patients, it is possible that PGHD was deglycosylated in the samples analyzed, resulting in the disappearance of acidic spots but no decrease in the total protein level.

Using capillary isoelectric focusing, Hiraoka and colleagues have identified changes in the charge microheterogeneity of CSF PGHD associated with various neurological disorders (Hiraoka A. *et al.*, *Electrophoresis* 2001, *22*, 3433-3437). The ratio of basic forms/acidic forms was found to increase in AD, in PD with pathological brain atrophy, and in multiple sclerosis. It was speculated that these post-translational modifications were linked to changes in the N-glycosylation pattern.

PGHD post-translational modifications (PTM) pattern in CSF of a Creutzfeldt-Jakob (CJD) disease patient:

We compared the PTM pattern of PGHD in CSF samples collected from a CJD patient and a healthy control. The proteins were separated by 2-DE, electroblotted on a PVDF membrane and PGHD was detected using a specific antibody, as

previously described. The CSF samples were collected by lumbar puncture. The control patient had a neurological workup for benign conditions unrelated to brain damage. CSF samples were centrifuged immediately after collection, aliquoted, frozen at -20°C and stored until analysis.

The results are shown in Figure 17 which is a comparison of prostaglandin D2 synthase spot intensities on mini-2-DE gels prepared either with CSF of a patient suffering from the Creutzfeldt-Jakob disease or with a control CSF from a healthy patient. Forty-five µg of protein were loaded on a IPG gel (pH 3-10 NL, 7 cm). Second dimension was a vertical gradient slab gel (12% T). Immunodetection was performed using an anti-human PGHD (lipocalin type) rabbit polyclonal antibody (Cayman chemical, Ann Arbor, MI) and ECLTM western blotting detection reagents (Amersham Biosciences, Uppsala, Sweden).

The results showed that the PTM pattern of PGHD in the CSF from the CJD patient is clearly different from the control, with a strong decrease of the 4 most acidic spots (Figure 17). The pattern of the CJD patient is similar to the one observed in post-mortem CSF. These data support the interest of changes in the PTM pattern of PGHD as marker of neurological disorders.

EXAMPLE 6

This Example provides additional data showing plasma levels of UFDP1 in stroke and control patients. Figure 19 shows the levels of UFDP1 in CSF of a control and a deceased patient. Additional data has been obtained from two cohorts of patients and controls, the smaller from Geneva, and a more comprehensive panel from the US. The methodology for this Example and following Examples 7 and 8 is the same, save that the antibodies being used have different specificities for the protein in question. The method in each of the studies is similar to that given as Example 4:

ELISA was performed using 96-well Reacti-Bind™ NeutrAvidin™ coated Black Plates (Pierce, Rockford, IL). Plates were first rinsed in Borate Buffer Saline pH 8.4 (BBS) (100 mM H3BO3, 25 mM Na2B4O7 (Sigma, St Louis,

MO, USA), 75 mM NaCl (Merck, Darmastadt, Germany)) on a NOVAPATH™ washer (Bio-Rad, Hercules, CA). Then, 50µl of relevant biomarker specific antibody-biotin conjugate (2 µg/mL) prepared in the dilution buffer A at pH 7 (DB, Polyvinyl Alcohol, 80% hydrolyzed, Mol. Wt. 9000-10,000 (Aldrich, Milwaukee, WI, USA), MOPS (3-[N-Morpholino] propane sulfonic acid) (Sigma), NaCl, MgCl2 (Sigma), ZnCl2 (Aldrich), pH6.90, BSA 30% Solution, Manufacturing Grade (Serological Proteins Inc., Kankakee, IL), were added and incubated for one hour at 37°C. Plates were then washed 3 times in BBS in the plate washer. 50 µl of antigen or plasma was then added and incubated for one hour at 37°C. Recombinant protein antigens were diluted at 100, 50, 25, 12.5, 6.25, 3.125, 1.56 ng/ml in the dilution buffer A to establish a calibration curve. Plasma samples were diluted at the appropriate dilution in the dilution buffer A. After a further washing step, 50µl of relevant biomarker specific alkaline phosphatase conjugated antibodies were added at the appropriate dilution in the dilution buffer A and incubated for one hour at 37°C. The 96-well plate was then washed 3 times with BBS in the plate washer and 50 µl of fluorescence Attophos® AP Fluorescent substrate (Promega, Madison, WI) were added. Plates were read immediately on a SpectraMax GEMINI-XS, (Molecular Devices Corporation, Sunnyvale, CA, U.S.A.) fluorometer microtiter plate reader

We read plates in fluorescence using a SpectraMax GEMINI-XS (Molecular Devices) fluorometer microplate reader ($\lambda_{excitation}$ =444 nm and $\lambda_{emission}$ =555 nm). Results are expressed in RFU and can be obtained in endpoint mode (only one reading) or in kinetic mode on 10 minutes. In kinetic mode, for each well we used 6 flashes (per well) which are integrated into an average and read each well 6 times using minimal interval time between each reading. This ends up being 2 minutes between readings. We determined a slope and this is what we used for our valuations. The best cut-off value to discriminate between the Control and the Stroke groups was determined by the ROC curves using GraphPad Prism 4 software.

The results are shown in Figure 20.

EXAMPLE 7

This corresponds to Example 6, except that the polypeptide is RNA-BP. Figure 21 shows the levels of RNA-BP in CSF of a control and a deceased patient. Figure 22 shows RNA-BP plasma concentration by ELISA for three studies, each comprising stroke patients and controls.

EXAMPLE 8

This corresponds to Example 6, except that the polypeptide is NDKA. Figure 23 shows the levels of NDKA in CSF of a control and a deceased patient. Figure 24 shows NDKA plasma concentration by ELISA for the Geneva and US cohorts of stroke patients and controls as in Example 6.

EXAMPLE 9

In addition to simple discrimination between stroke and control patients, the data from each of Examples 6, 7 and 8 can be analysed in relation to the time between cerebrovascular accident and sample collection, or alternatively in relation to the type of stroke – ischaemic, haemorrhagic or transient ischaemic attack (TIA). These separate analyses are shown in Figure 25a and Figure 25b and demonstrate the utility of deceased CSF markers in the diagnosis of stroke. This is particularly relevant to clinical practice as it is essential to diagnose stroke within three hours of the event to allow administration of clot busting drugs such as TPA. It is also essential that tests can differentiate haemorrhagic stroke from ischaemic attack as TPA is only suitable for the treatment of ischaemia and can have catastrophic effects in patients with an haemorrhagic stroke.

EXAMPLE 10

Whilst each of the deceased CSF markers have good individual performance for the diagnosis of stroke, it is likely that a commercial product will require the measurement of levels of several proteins. This 'panel' approach can be achieved

in two ways. In the simpler approach the antibodies for each separate marker are pooled and used to coat microtitre wells. The intensity of the signal will be the sum of that for each independent marker, though in this case it will be impossible to determine the individual levels of each of the markers. This may create challenges in setting meaningful cut-off values, however, this presents the most user friendly commercial product.

Figure 26 summarises the markers which are used in this Example. Experimental results are shown in Figure 27, in which antibodies against the deceased CSF proteins UFD1, RNA-BP, NDKA and H-FABP were used at the same concentrations as in Example 4. However, these antibody solutions were mixed in equal volumes, reducing the concentration of each antibody species to one quarter of the original level in the single analyte examples described above. The protocol used is as follows:

To overcome the problem of panel algorithm, we tested the four antibodies directly in mixture in each well. The protocol is exactly the same as previously described for separated antibodies (above), save that each of the biomarker specific biotin-antibody conjugates were used at 12.5 µL per well during the first antibody coating step. The standard curve was similarly constructed by using 12.5 µL per well of each of the four recombinant protein antigens UFDP1, RNA-BP, NDKA and H-FABP each prepared separately at initial concentrations of 100, 50, 25, 12.5, 6.25, 3.125, 1.56 ng/ml in the dilution buffer A to establish a calibration curve on the same plate. Plasma samples were used at the same dilution and volume (50 µL per well) as for the individual biomarker assays. Detection of captured antigens was performed using the same biomarker specific antibody-alkaline phosphatases conjugates as the individual assays, with equal volumes (12.5 µL) of the four biomarker specific antibody-alkaline phosphatases conjugates being added to each well for the standard curve and plasma samples. Measurement of fluorescence was performed as described for the single biomarker assays as described in the example above.

Ten stroke and ten control (non age/sex matched) plasma samples 2-fold diluted were tested (Figure 27). This experiment led to 100% sensitivity and 80% specificity. The two false positives samples correspond to patient's control 368 and 450 that display prostate cancer and probable head trauma.

In this specific example the fluorescence signal obtained corresponds to the sum of the signal generated by each biomarker specific antibody sandwich and it is impossible to determine the relative contribution of each single biomarker to the whole when using alkaline-phosphatase conjugated antibodies for the detection side of the assay. It is also an aspect of the invention that each biomarker specific antibody can be labelled with a different fluorophore with sufficient difference between their excitation and emission wavelengths that the level of each antibody can be determined without interference. In this case it is possible to accurately quantify the levels of up to four different biomarkers in a sample in a single assay, providing benefits in reduced sample requirement and increased throughput.

EXAMPLE 11

In some circumstances it may not be desirable to measure levels of multiple analytes in a single well. For example the absolute levels of individual proteins, or the ratio between levels of multiple proteins may be necessary to make a specific diagnosis. In this situation it may be desirable to measure the levels of each analyte in a separate assay. A predictive algorithm is then used to interpret these multiparametric datasets to provide a unique diagnosis for each patient. In this Example we have used a statistical algorithm to predict the theoretical performance of different multi-analyte biomarker panels.

The datasets of individual biomarker levels generated in the various examples above were analysed using a proprietary algorithm to determine the true positive and true negative rates for each combination of the deceased CSF proteins UFDP1, RNA-BP, NDKA and H-FABP for the diagnosis of stroke. For the analysis a Sample set (18 controls and 18 stroke for UFD1, RNA-BP, NDK A and H-FABP) was divided into 2 random populations.

80% of the total samples for training of the thresholds was performed by the technique of naive bayes, and the remaining 20 % of the total samples were then used to evaluate the thresholds (sensitivity and specificity) for each marker, or combination of markers made 1000 fold.

Where the algorithm was applied to single proteins it was possible to compare sensitivities and specificities values with those observed. The sensitivity and specificity for these data sets (figures in parentheses) were calculated based on the optimum cut-off determined from the ROC curve as described in the examples above. In the following data, the first value in parenthesis corresponds to standard deviation (e.g. 0.93 $\pm$ 0.15). The second value in parenthesis for the "1 protein" data corresponds to sensitivity (SE) and specificity (SP) obtained without using the algorithm, but using simple ROC curve (GraphPad Prism). The SE and SP values are indicated just to compare the results with and without the algorithm.

The output of this algorithm analysis was as follows:

1 protein

True positive rate of UFD1 on training set: 0.93 (0.15) (SE 94%)
True negative rate of UFD1 on training set: 0.74 (0.24) (SP 78%)
True positive rate of RNA-BP on training set: 0.85 (0.21) (SE 94%)
True negative rate of RNA-BP on training set: 0.73 (0.23) (SP 72%)
True positive rate of H-FABP on training set: 0.47 (0.29) (SE 39%)
True negative rate of H-FABP on training set: 0.80 (0.23) (SP 100%)
True positive rate of NDK A on training set: 0.79 (0.24) (SE 100%)
True negative rate of NDK A on training set: 0.89 (0.16) (SP 83%)

2 proteins

True positive rate of UFD1/RNA-BP on training set: 0.90 (0.17)
True negative rate of UFD1/RNA-BP on training set: 0.69 (0.25)
True positive rate of UFD1/H-FABP on training set: 0.82 (0.22)
True negative rate of UFD1/H-FABP on training set: 0.83 (0.20)
True positive rate of UFD1/NDK A on training set: 0.92 (0.16)

True negative rate of UFD1/NDK A on training set: 0.79 (0.21)
True positive rate of RNA-BP/H-FABP on training set: 0.81 (0.24)
True negative rate of RNA-BP/H-FABP on training set: 0.73 (0.24)
True positive rate of RNA-BP/NDK A on training set: 0.91 (0.16)
True negative rate of RNA-BP/NDK A on training set: 0.83 (0.21)
True positive rate of H-FABP/NDK A on training set: 0.77 (0.27)
True negative rate of H-FABP/NDK A on training set: 0.84 (0.20)

3 proteins

True positive rate of RNA-BP/NDK A/H-FABP on training set: 0.96 (0.11)
True negative rate of RNA-BP/NDK A/H-FABP on training set: 0.83 (0.20)

True positive rate of UFD1/NDK A/H-FABP on training set: 0.92 (0.17)
True negative rate of UFD1/NDK A/H-FABP on training set: 0.83 (0.21)

True positive rate of UFD1/RNA-BP/NDKA on training set: 0.95 (0.14)
True negative rate of UFD1/RNA-BP/NDKA on training set: 0.82 (0.20)

True positive rate of UFD1/RNA-BP/H-FABP on training set: 0.93 (0.15)
True negative rate of UFD1/RNA-BP/HFABP on training set: 0.75 (0.23)

The 4 proteins

True positive rate of UFD1/RNA-BP/H-FABP/NDK A on training set: 0.93 (0.13)
True negative rate of UFD1/RNA-BP/H-FABP/NDK A on training set: 0.73 (0.23)

Figure 28 is a graphical representation of combinations of two out of the four biomarkers used in this Example. It shows the cut-off points (horizontal and vertical lines) which we have determined for diagnosis.

EXAMPLE 12

Further large scale studies were performed in Geneva and USA on UFD1, RNA-BP and NDK A post mortem CSF markers. ELISA was carried out on samples as described in the previous Examples (both for the Geneva as well as the USA experiments). The results are shown in Figures 29-38.

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CLAIMS

1 A method of diagnosis of a brain damage-related disorder or the possibility thereof in a subject suspected of suffering therefrom, which comprises detecting at least one polypeptide, or a variant or mutant thereof, selected from A-FABP, E-FABP, H-FABP, B-FABP, PGP 9.5, GFAP, Prostaglandin D synthase, Neuromodulin, Neurofilament L, Calcyphosine, RNA binding regulatory subunit, Ubiquitin fusion degradation protein 1 homolog, Nucleoside diphosphate kinase A, Glutathione S tranferase P, Cathepsin D, DJ-1 protein, Peroxiredoxin 5 and Peptidyl-prolyl cis-trans isomerase A (Cyclophilin A) in a sample of body fluid taken from the subject.

2 A method according to Claim 1, in which the polypeptide is differentially contained in the body fluid of brain damage-related disorder-affected subjects and non-brain damage-related disorder-affected subjects, and the method includes determining whether the concentration of polypeptide in the sample is consistent with a diagnosis of brain damage-related disorder.

3 A method according to Claim 1 or 2, in which an antibody to the polypeptide is used in the detection or the determination of the concentration.

4 A method according to any of Claims 1 to 3, in which the body fluid is cerebrospinal fluid, plasma, serum, blood, tears, urine or saliva.

5 A method according to any of Claims 1 to 4, in which the polypeptide is present in the body fluid of brain damage-related disorder-affected subjects and not present in the body fluid of non-brain damage-related disorder-affected subjects, whereby the presence of the polypeptide in a body fluid sample is indicative of brain damage-related disorder.

6 A method according to any of Claims 1 to 4, in which the polypeptide is not present in the body fluid of brain damage-related disorder-affected subjects and present in the body fluid of non-brain damage-related disorder-affected

subjects, whereby the non-presence of the polypeptide in a body fluid sample is indicative of brain damage-related disorder.

7 A method according to any of Claims 1 to 6, in which a plurality of peptides is determined in the sample.

8 A method according to any of Claims 1 to 7, in which the polypeptide is differentially subject to post-translational modification in the body fluid of brain damage-related disorder-affected subjects and non-brain damage-related disorder-affected subjects, and the method includes detecting the post-translational modification of the polypeptide in the sample and determining whether this is consistent with a diagnosis of a brain damage-related disorder.

9 A method according to Claim 8, in which the post-translational modification comprises N-glycosylation.

10 A method according to any of Claims 1 to 9, in which the brain damage-related disorder is stroke and the polypeptide is Ubiquitin fusion degradation protein 1 homolog.

11 A method according to any of Claims 1 to 9, in which the brain damage-related disorder is stroke and the polypeptide is RNA binding regulatory subunit.

12 A method according to any of Claims 1 to 9, in which the brain damage-related disorder is stroke and the polypeptide is Nucleoside diphosphate kinase A.

13 A method according to any of Claims 10 to 12, in which two or more markers selected from antibodies to Ubiquitin fusion degradation protein 1 homolog, RNA binding regulatory subunit, Nucleoside diphosphate kinase A and H-FABP are used in a single well of an ELISA microtiter plate.

14 A method according to Claim 13, in which all four markers are used in a single well.

15 A method according to any of Claims 10 to 12, in which two or more polypeptides selected from Ubiquitin fusion degradation protein 1 homolog, RNA binding regulatory subunit, Nucleoside diphosphate kinase A and H-FABP are separately assayed, and a predictive algorithm is used for diagnosis.

16 Use of a polypeptide, or a variant or mutant thereof, selected from A-FABP, E-FABP, H-FABP, B-FABP, PGP 9.5, GFAP, Prostaglandin D synthase, Neuromodulin, Neurofilament L, Calcyphosine, RNA binding regulatory subunit, Ubiquitin fusion degradation protein 1 homolog, Nucleoside diphosphate kinase A, Glutathione S tranferase P, Cathepsin D, DJ-1 protein, Peroxiredoxin 5 and Peptidyl-prolyl cis-trans isomerase A (Cyclophilin A), or a combination of such polypeptides, for diagnostic, prognostic and therapeutic applications relating to brain damage-related disorders.

17 Use according to Claim 16, in which the polypeptide is differentially contained in a body fluid of brain damage-related disorder-affected subjects and non-brain damage-related disorder-affected subjects.

18 Use for diagnostic, prognostic and therapeutic applications, relating to brain damage-related disorders, of a material which recognises, binds to or has affinity for a polypeptide, or a variant or mutant thereof, selected from A-FABP, E-FABP, H-FABP, B-FABP, PGP 9.5, GFAP, Prostaglandin D synthase, Neuromodulin, Neurofilament L, Calcyphosine, RNA binding regulatory subunit, Ubiquitin fusion degradation protein 1 homolog, Nucleoside diphosphate kinase A, Glutathione S tranferase P, Cathepsin D, DJ-1 protein, Peroxiredoxin 5 and Peptidyl-prolyl cis-trans isomerase A (Cyclophilin A).

19 Use according to Claim 18 of a combination of materials, each of which respectively recognises, binds to or has affinity for a polypeptide, or a variant or mutant thereof, selected from A-FABP, E-FABP, H-FABP, B-FABP, PGP 9.5, GFAP, Prostaglandin D synthase, Neuromodulin, Neurofilament L, Calcyphosine, RNA binding regulatory subunit, Ubiquitin fusion degradation protein 1 homolog, Nucleoside diphosphate kinase A, Glutathione S tranferase P, Cathepsin D, DJ-1

protein, Peroxiredoxin 5 and Peptidyl-prolyl cis-trans isomerase A (Cyclophilin A).

20 Use according to Claim 18 or 19, in which the or each material is an antibody or antibody chip.

21 Use according to Claim 20, in which the material is an antibody to A-FABP.

22 Use according to Claim 20, in which the material is an antibody to E-FABP.

23 Use according to Claim 20, in which the material is an antibody to PGP 9.5.

24 Use according to Claim 20, in which the material is an antibody to GFAP.

25 Use according to Claim 20, in which the material is an antibody to Prostaglandin D synthase.

26 Use according to Claim 20, in which the material is an antibody to Neuromodulin.

27 Use according to Claim 20, in which the material is an antibody to Neurofilament L.

28 Use according to Claim 20, in which the material is an antibody to Calcyphosine.

29 Use according to Claim 20, in which the material is an antibody to RNA binding regulatory subunit.

30 Use according to Claim 20, in which the material is an antibody to Ubiquitin fusion degradation protein 1 homolog.

31 Use according to Claim 20, in which the material is an antibody to Nucleoside diphosphate kinase A.

32 Use according to Claim 20, in which the material is an antibody to Glutathione S tranferase P.

33 Use according to Claim 20, in which the material is an antibody to Cathepsin D.

34 Use according to Claim 20, in which the material is an antibody to DJ-1 protein.

35 Use according to Claim 20, in which the material is an antibody to Peroxiredoxin 5.

36 Use according to Claim 20, in which the material is an antibody to Peptidyl-prolyl cis-trans isomerase A (Cyclophilin A).

37 An assay device for use in the diagnosis of brain damage-related disorders, which comprises a solid substrate having a location containing a material which recognizes, binds to or has affinity for a polypeptide, or a variant or mutant thereof, selected from A-FABP, E-FABP, H-FABP, B-FABP, PGP 9.5, GFAP, Prostaglandin D synthase, Neuromodulin, Neurofilament L, Calcyphosine, RNA binding regulatory subunit, Ubiquitin fusion degradation protein 1 homolog, Nucleoside diphosphate kinase A, Glutathione S tranferase P, Cathepsin D, DJ-1 protein, Peroxiredoxin 5 and Peptidyl-prolyl cis-trans isomerase A (Cyclophilin A).

38 An assay device according to Claim 37, in which the solid substrate has a plurality of locations each respectively containing a material which recognizes, binds to or has affinity for a polypeptide, or a variant or mutant thereof, selected from A-FABP, E-FABP, H-FABP, B-FABP, PGP 9.5, GFAP, Prostaglandin D synthase, Neuromodulin, Neurofilament L, Calcyphosine, RNA binding regulatory

subunit, Ubiquitin fusion degradation protein 1 homolog, Nucleoside diphosphate kinase A, Glutathione S tranferase P, Cathepsin D, DJ-1 protein, Peroxiredoxin 5 and Peptidyl-prolyl cis-trans isomerase A (Cyclophilin A).

39 An assay device according to Claim 37 or 38, in which the material is an antibody or antibody chip.

40 An assay device according to Claim 39, which has a unique addressable location for each antibody, thereby to permit an assay readout for each individual polypeptide or for any combination of polypeptides.

41 An assay device according to any of Claims 37 to 40, including an antibody to A-FABP.

42 An assay device according to any of Claims 37 to 40, including an antibody to E-FABP.

43 An assay device according to any of Claims 37 to 40, including an antibody to PGP 9.5.

44 An assay device according to any of Claims 37 to 40, including an antibody to GFAP.

45 An assay device according to any of Claims 37 to 40, including an antibody to Prostaglandin D synthase.

46 An assay device according to any of Claims 37 to 40, including an antibody to Neuromodulin.

47 An assay device according to any of Claims 37 to 40, including an antibody to Neurofilament L.

48 An assay device according to any of Claims 37 to 40, including an antibody to Calcyphosine.

49 An assay device according to any of Claims 37 to 40, including an antibody to RNA binding regulatory subunit.

50 An assay device according to any of Claims 37 to 40, including an antibody to Ubiquitin fusion degradation protein 1 homolog.

51 An assay device according to any of Claims 37 to 40, including an antibody to Nucleoside diphosphate kinase A.

52 An assay device according to any of Claims 37 to 40, including an antibody to Glutathione S tranferase P.

53 An assay device according to any of Claims 37 to 40, including an antibody to Cathepsin D.

54 An assay device according to any of Claims 37 to 40, including an antibody to DJ-1 protein.

55 An assay device according to any of Claims 37 to 40, including an antibody to Peroxiredoxin 5.

56 An assay device according to any of Claims 37 to 40, including an antibody to Peptidyl-prolyl cis-trans isomerase A (Cyclophilin A).

57 A kit for use in the diagnosis of brain damage-related disorders, comprising an assay device according to any of Claims 37 to 56, and means for detecting the amount of one or more of the polypeptides in a sample of body fluid taken from a subject.

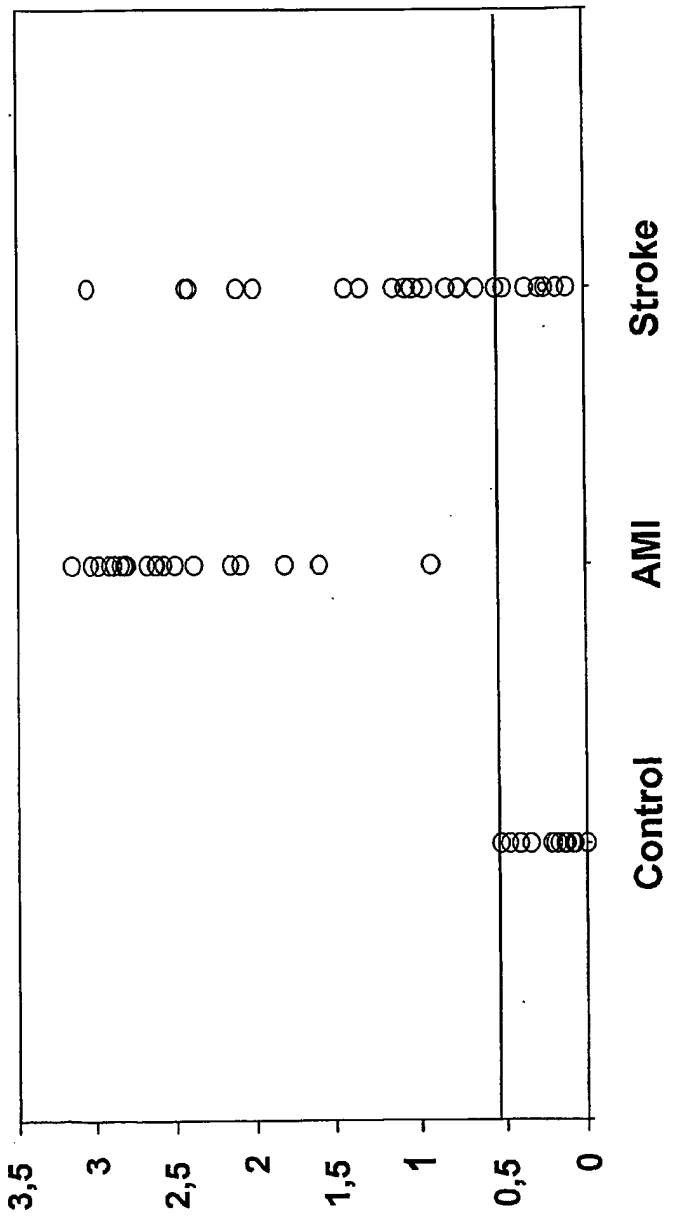


Figure 1

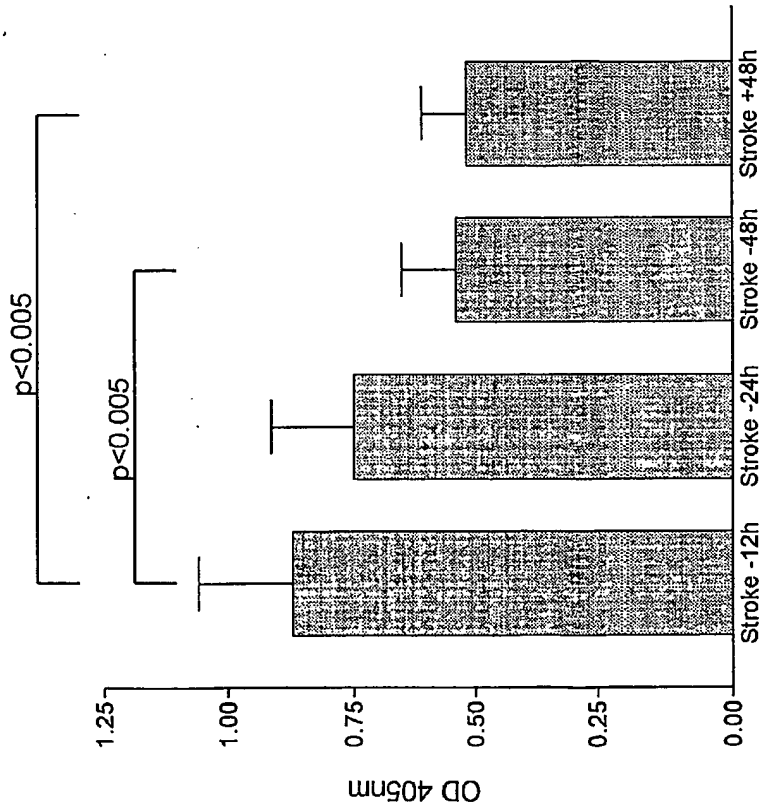

p<0.005
p<0.005
Stroke -12h
Stroke -24h
Stroke -48h
Stroke +48h
1.25
1.00
0.75
0.50
0.25
0.00
OD 405nm

Figure 2

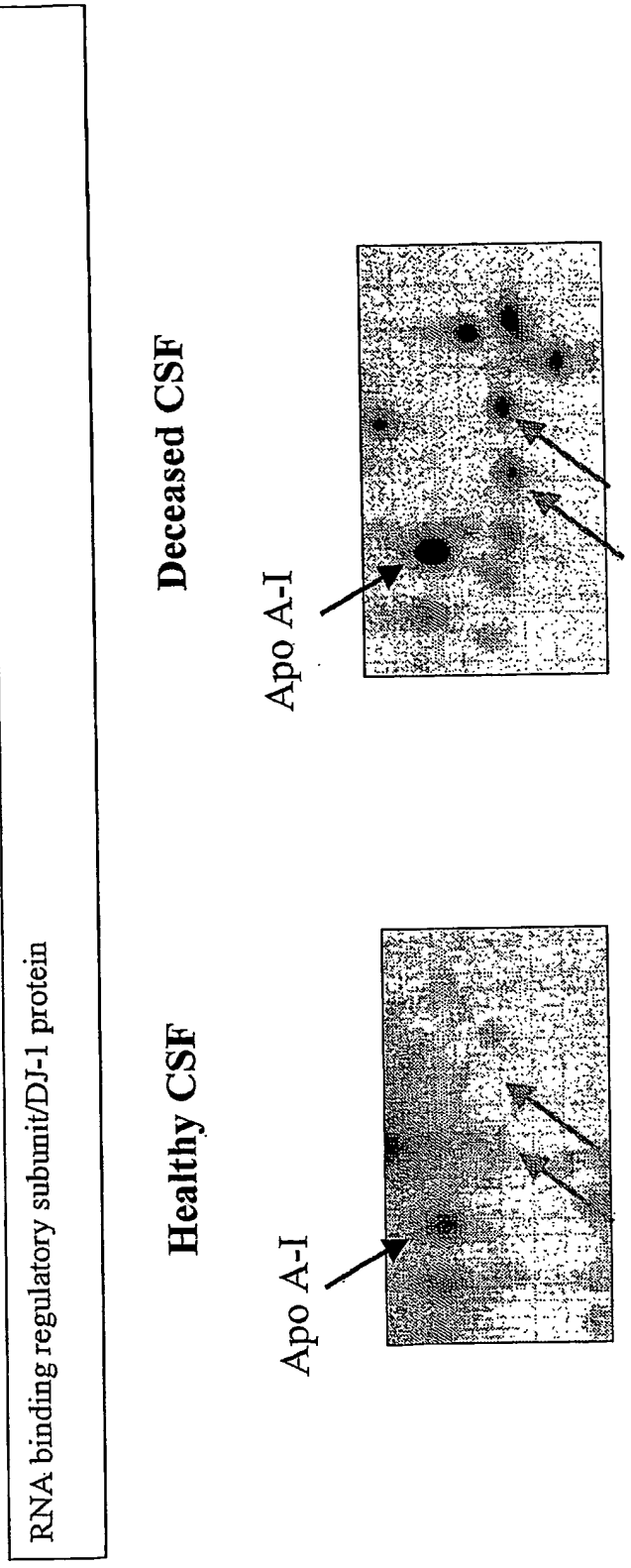

RNA binding regulatory subunit/DJ-1 protein
Healthy CSF
Apo A-I
Deceased CSF
Apo A-I

Figure 3

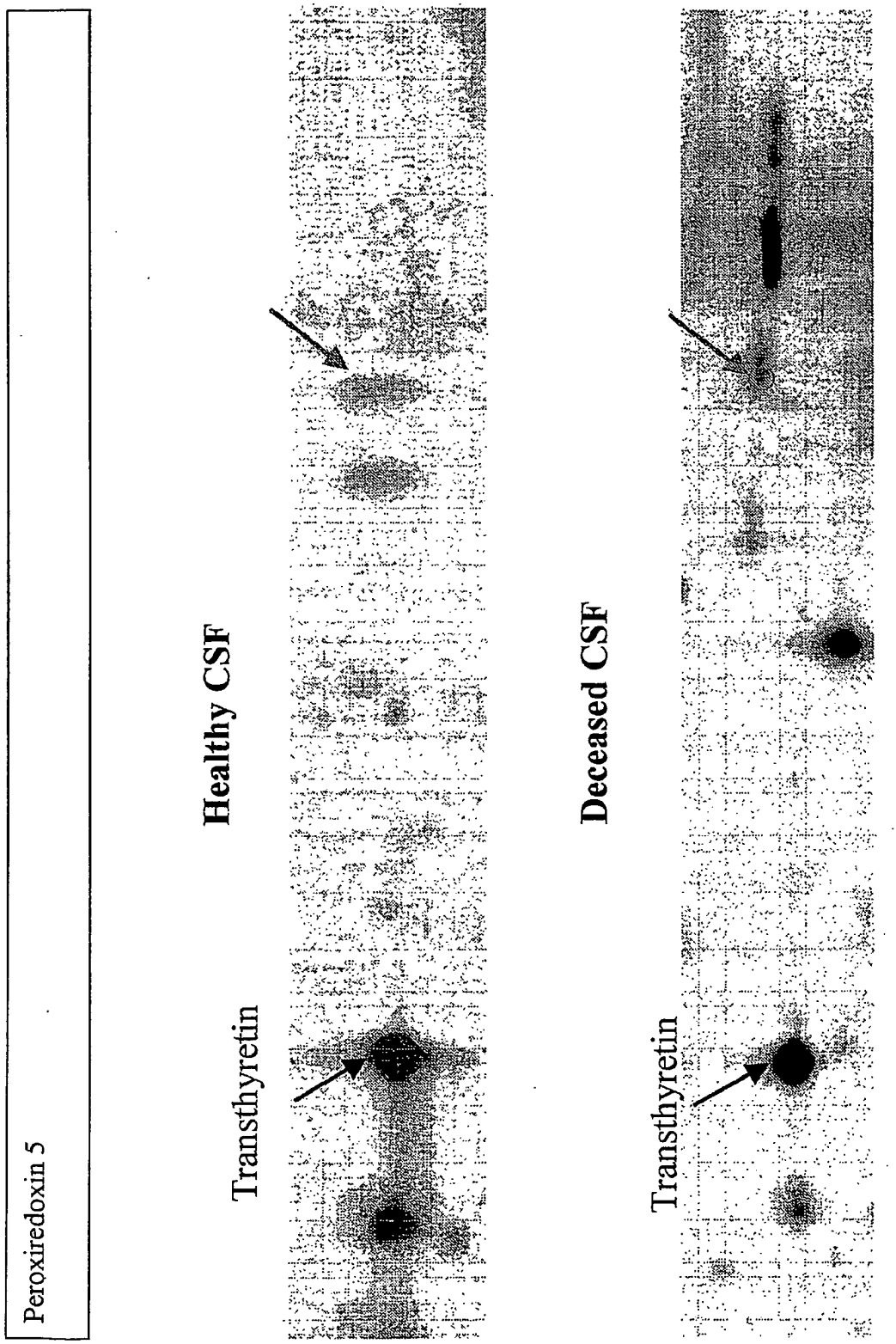

Peroxiredoxin 5
Healthy CSF
Transthyretin
Deceased CSF
Transthyretin

Figure 4

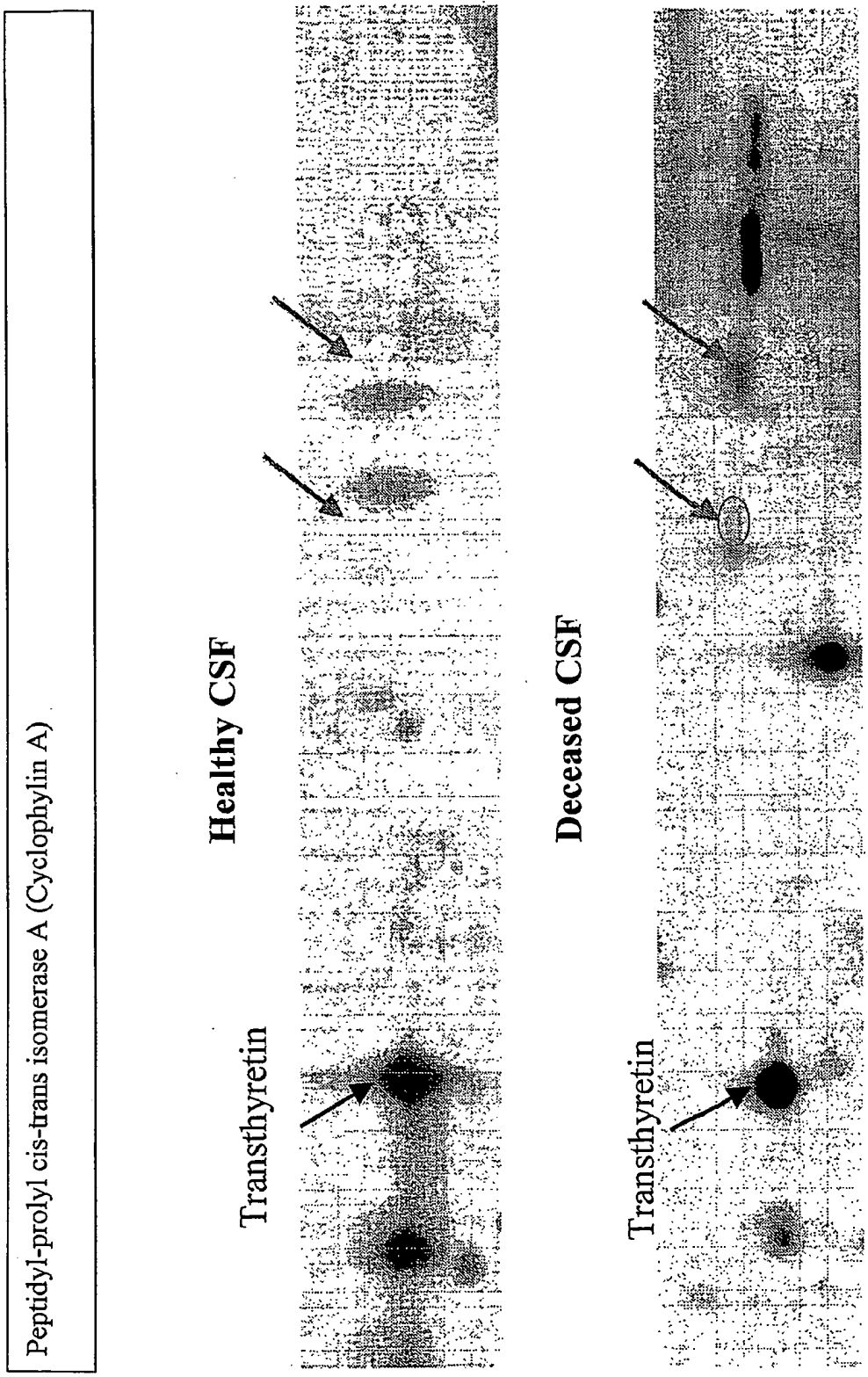

Peptidyl-prolyl cis-trans isomerase A (Cyclophylin A)
Healthy CSF
Transthyretin
Deceased CSF
Transthyretin

Figure 5

Figure 6: ELISA intensity signal obtained for UFD1, RNA-BP and NDK A

stroke patients matched age/sex with control patients

STROKE PATIENTS (between 0-24h arrival at emergency hospital)									NEGATIVE CONTROL PATIENTS					
Patient number	Diagnostic	Sex	year of birth	time onset of symptoms	time onset of symptoms (min)	UFD1 (RFU signal) CO=9047	RNA-BP (RFU signal) CO=7441	NDK A (RFU signal) CO=12500	Patient number	sex	year of birth	UFD1 (RFU signal)	RNA-BP (RFU signal)	NDK A (RFU signal) CO=12500
186	Ic	M	1931	30 min	30	7127	10844	13,639	368	M	1931	10365	12267	75,072
253	Irr	F	1975	45min	45	39636	14367	19,907	401	F	1972	1306	1209	2,398
245	Ic	M	1925	1h15	75	10900	11444	38,160	404	M	1925	3564	4525	7,425
243	H	M	1938	1h18	78	21008	22046	25,508	388	M	1938	2643	3867	11,877
239	TIA	M	1923	1h40	100	17122	7471	37,548	464	M	1923	2957	5775	6,292
202	H	M	1949	1h15	75	12225	8379	66,554	305	M	1949	37188	4587	5,449
229	H	M	1932	2h05	125	9237	14931		317	M	1931	8857	13370	7,183
271	Irr	M	1913	2h07	127	11658	27199	22,313	439	M	1913	4248	4348	11,884
256	TIA	F	1935	3h00	180	17727	23110	20,671	378	F	1935	3512	4420	7,548
267	Ic	M	1928	3h00	180	25665	11309	69,539	339	M	1929	2455	3784	5,086
208	Irr	F	1945	8h00	480	12617	20467	13,080	349	F	1946	4076	4103	5,166
212	Irr	M	1934	10h30	630	27326	11986	17,216	379	M	1934	8791	8751	10,497
258	Ic	M	1920	1 d	1440	16814	9392	26,118	400	M	1922	4919	7411	5,920
234	TIA	M	1914	2 d	2880	22273	13278	78,373	322	M	1915	5373	5757	12,112
246	Ic	M	1920	2 d	2880	10374	10083	27,109	443	M	1919	11589	13479	13,797
250	Ic	M	1908	4 d	5760	32857	5702	122,914	450	M	1909	17357	12344	47,866
240	Irr	M	1926	5 d	7200	9796	17691	12,817	430	M	1926	2660	4505	7,542
254	Irr	F	1960	?	?	21142	10078	29,784	354	F	1955	3711	3647	6,360
249	Irr							32,639						
STROKE PATIENTS (after 72h arrival at emergency hospital)														
239	TIA	M	1923	1h40	100	11517	7169	19918						
202	H	M	1949	1h15	75	5764	7706	27685						
299	Ic					16357	11919	21931						

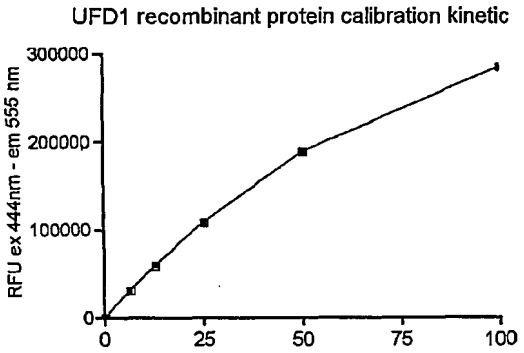
Ic: established stroke
Irr: Ischemic rapidly resolved
TIA: transient ischemic attack
H: Hemorrhagic
M: Male
F: Female
RFU: Relative Fluorescence Unit (excitation wavelength 444nm, emission wavelength 555nm)

positive plasma (/ cutoff)

patient tested between 0-24h AND after 72h

25,508 patient (Hemorrhagic) n°273 age/sex matched with the control instead of n° 243

Figure 7. UFD1 detection in new plasma samples 2 fold diluted. Antibodies sandwich immunofluorescent ELISA. Crude values kinetic mode. Controls/stroke matched age/sex



Rec UFD1	RFU
100 ng/mL	284423.5
50 ng/mL	189477.5
25 ng/mL	109317.5
12.5 ng/mL	60429.5
6.25 ng/mL	32226
0	687.015

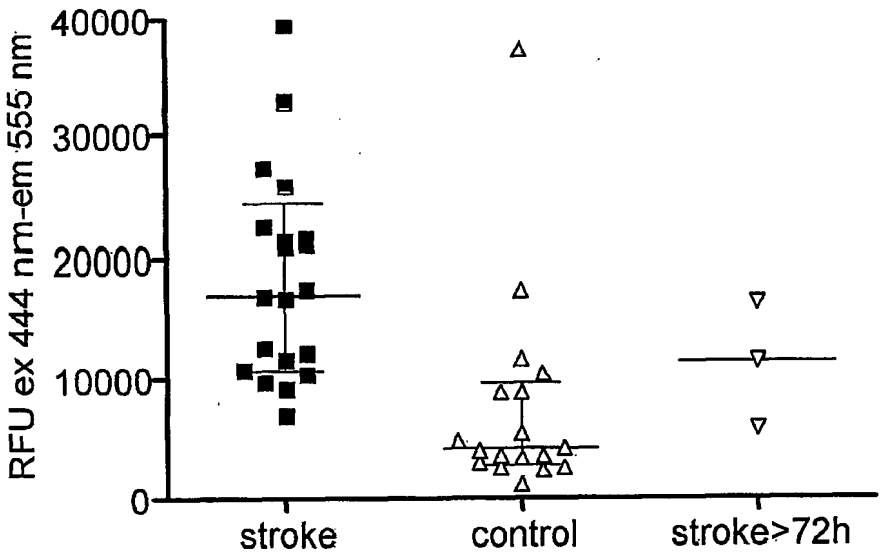
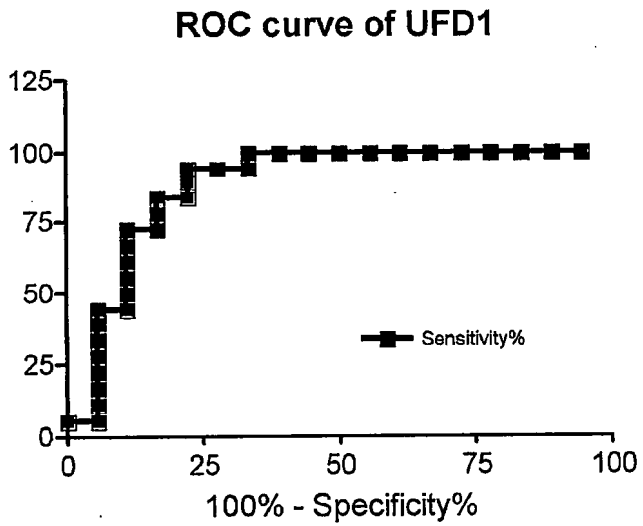


Figure 8. ROC curve of UFD1



UFD1 best cutoff value to differentiate stroke vs control. Determination of sensitivity and specificity

	cutoff	P (Mann et Whitney)	SE	SP
S vs. C	9047	<0.0001	94.4%	77.8%

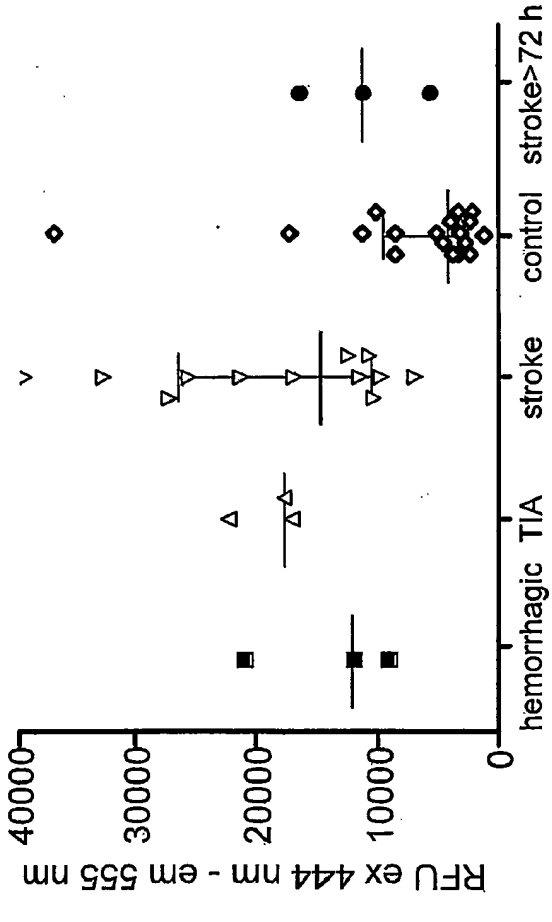
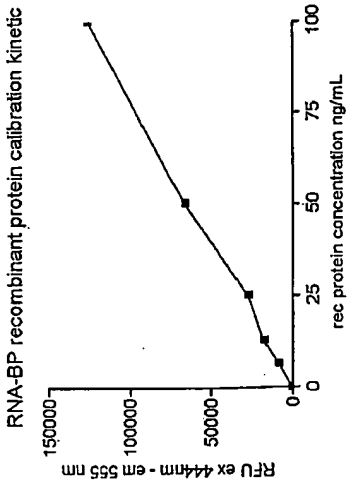


Figure 9. UFD1 detection in new plasma samples 2 fold diluted. Antibodies sandwich immunofluorescent ELISA. Crude values kinetic mode. Controls/stroke matched age/sex

Figure 10. RNA-BP detection in new plasma samples 2 fold diluted. Antibodies sandwich immunofluorescent ELISA. Crude values kinetic mode. Controls/stroke matched age/sex



Rec RNA-BP	RFU
100ng/ml	126449.5
50ng/ml	65653.5
25ng/ml	27935
12.5ng/ml	18315
6.25ng/ml	9185.25
0ng/ml	940.97

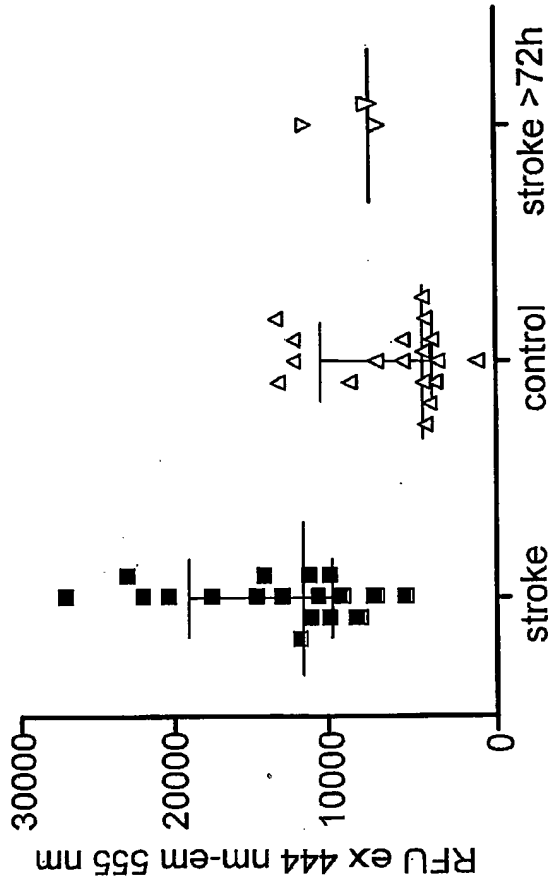
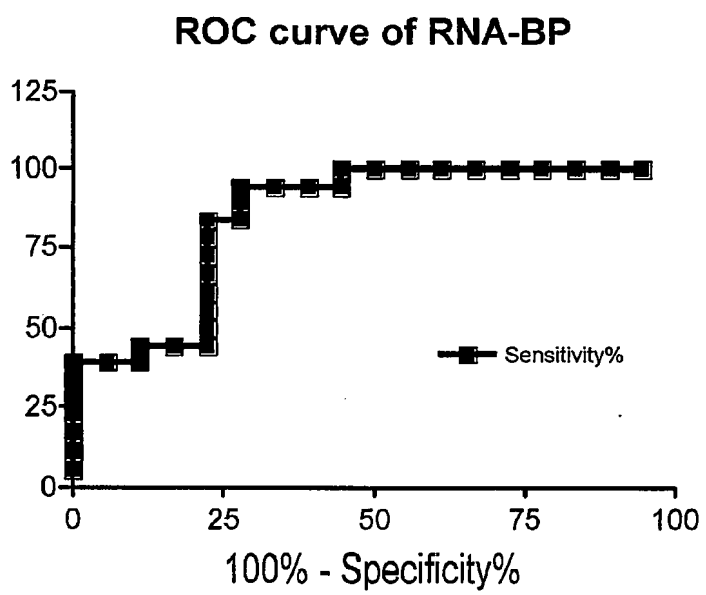


Figure 11. ROC curve of RNA-BP



RNA-BP best cutoff value to differentiate stroke vs control. Determination of sensitivity and specificity

	cutoff	P (Mann et Whitney)	SE	SP
S vs. C	7441	0.0003	94.4%	72.2%

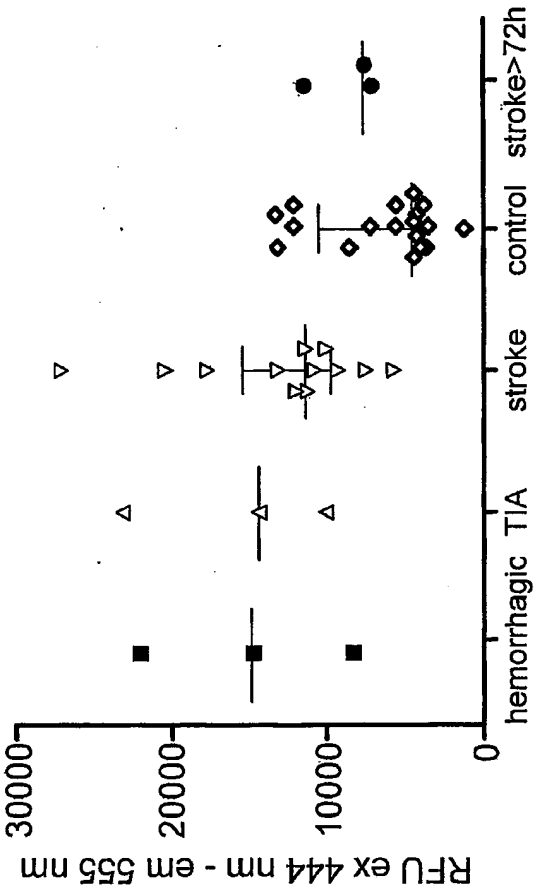


Figure 12. RNA-BP detection in new plasma samples 2 fold diluted. Antibodies sandwich immunofluorescent ELISA. Crude values kinetic mode. Controls/stroke matched age/sex

Figure 13. NDK A detection in new plasma samples non diluted. Antibodies sandwich immunofluorescent ELISA. Crude values kinetic mode. Controls/stroke matched age/sex

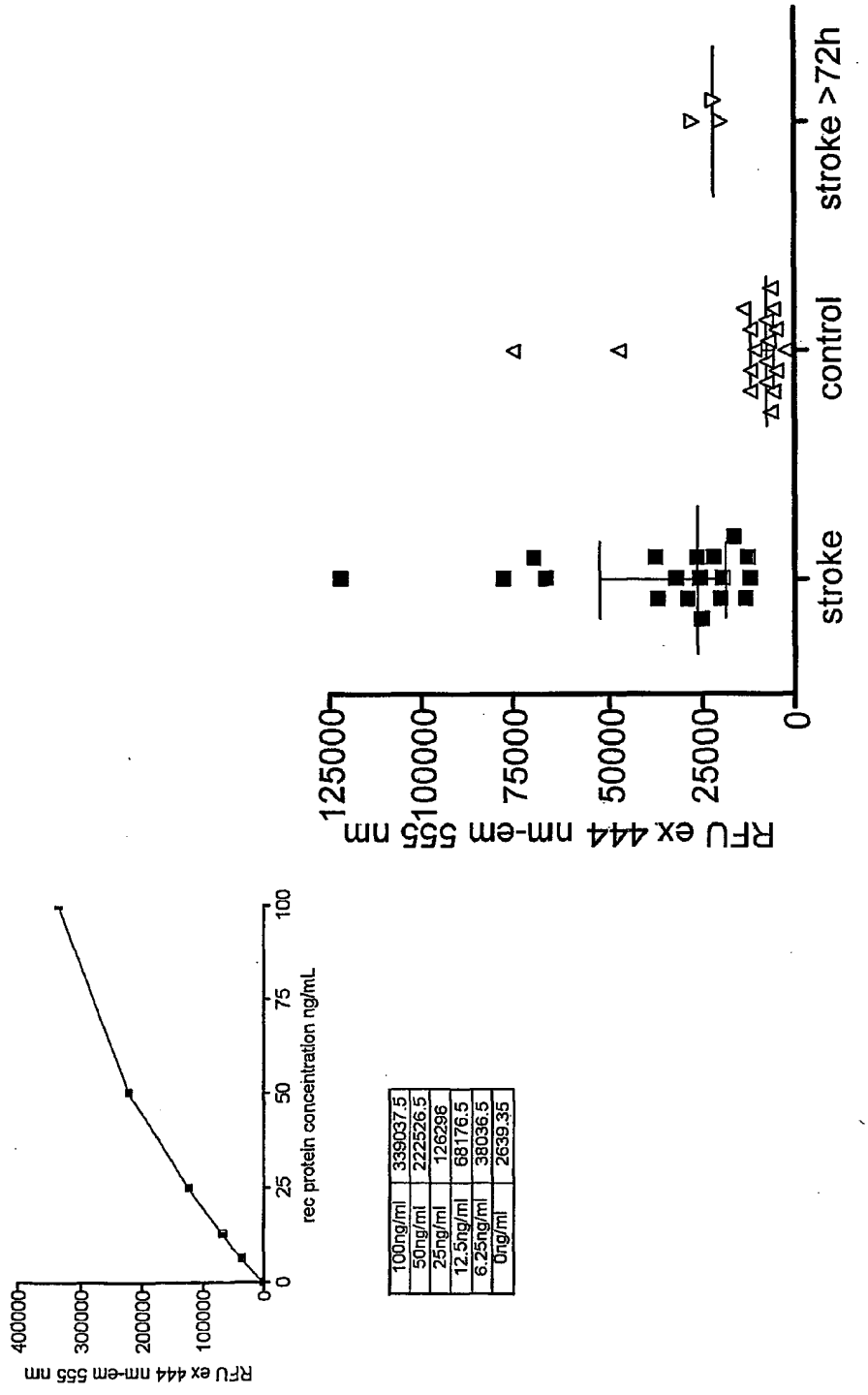
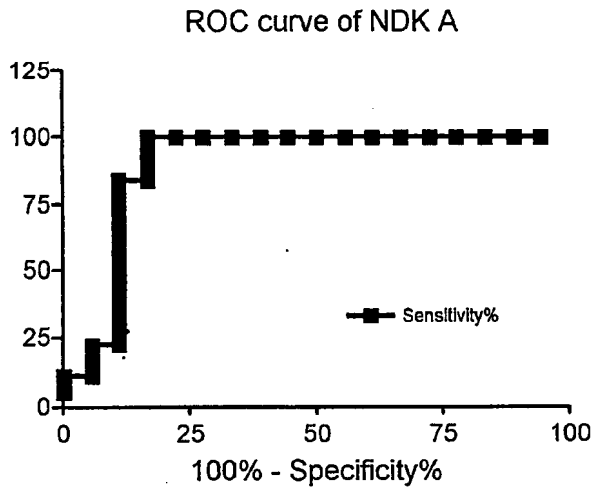


Figure 14. ROC curve of NDK A



NDK A best cutoff value to differentiate stroke vs control. Determination of sensitivity and specificity

	cutoff	P (Mann et Whitney)	SE	SP
S vs. C	12464	<0.0001	100%	83.3%

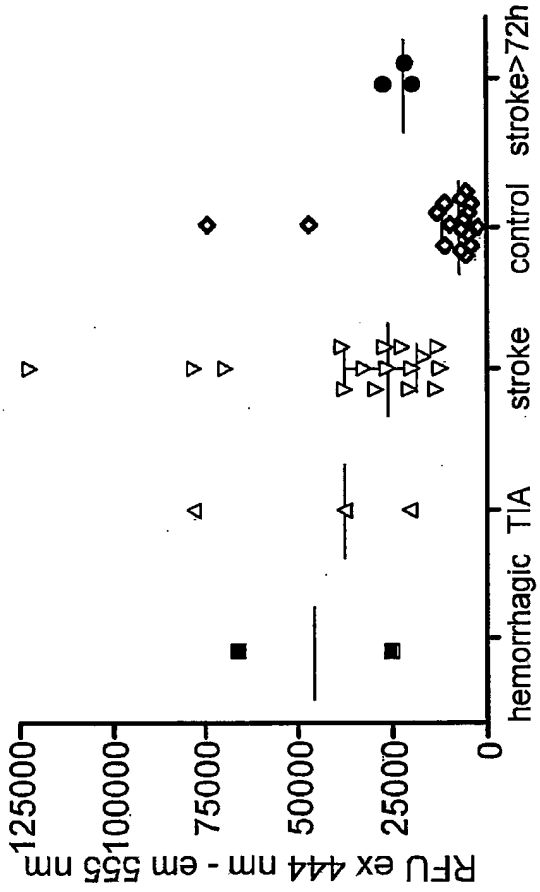


Figure 15. NDK A detection in new plasma samples non diluted. Antibodies sandwich immunofluorescent ELISA. Crude values kinetic mode. Controls/stroke matched age/sex

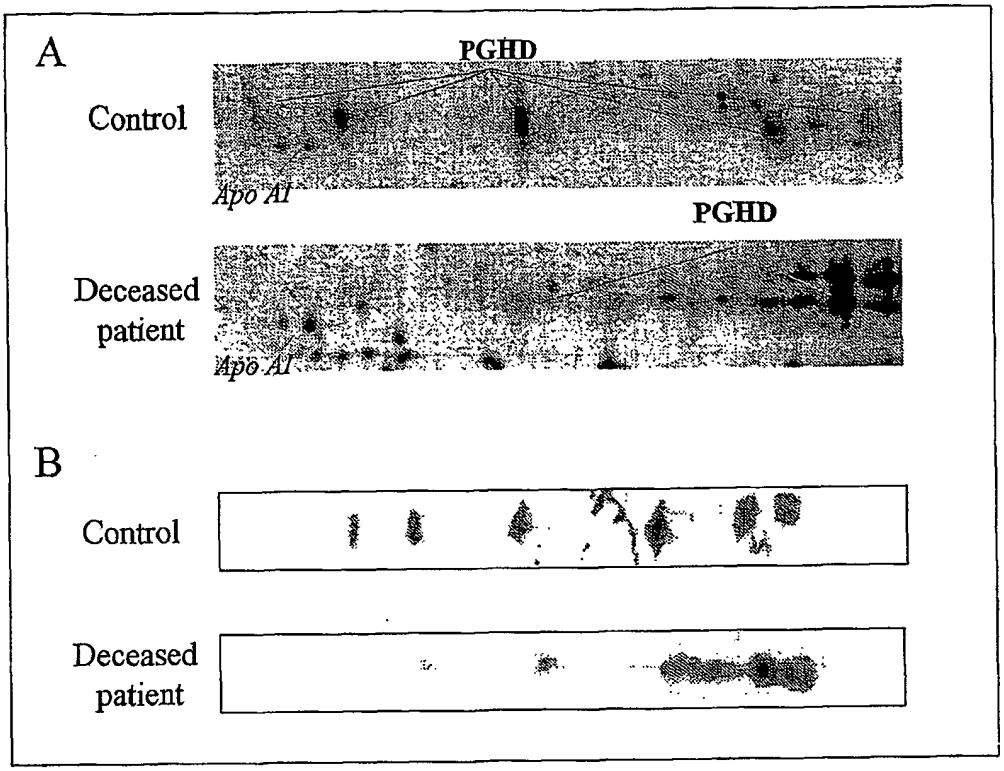
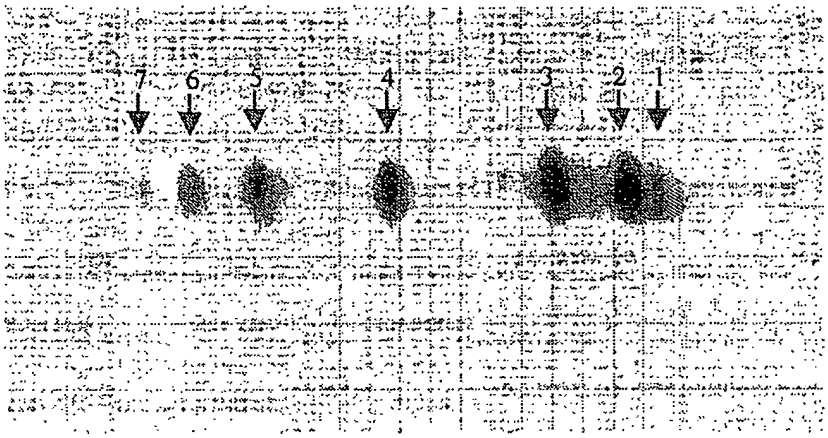

A
PGHD
Control
Apo AI
PGHD
Deceased patient
Apo AI
B
Control
Deceased patient

Figure 16

Control



CJD patient

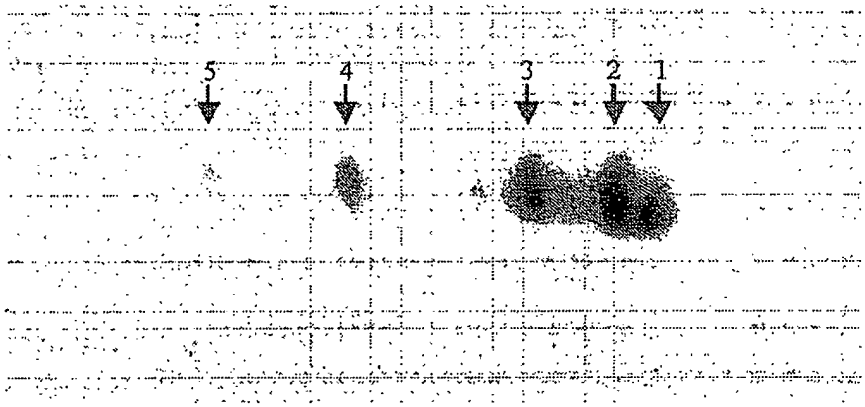
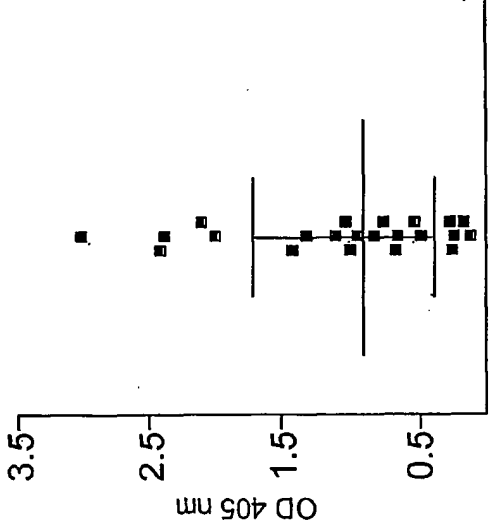


Figure 17

Figure 18 - Heart-Fatty Acid Binding Protein (H-FABP)



	p	SE	SP
S vs. C	< 0.0001	68%	100%

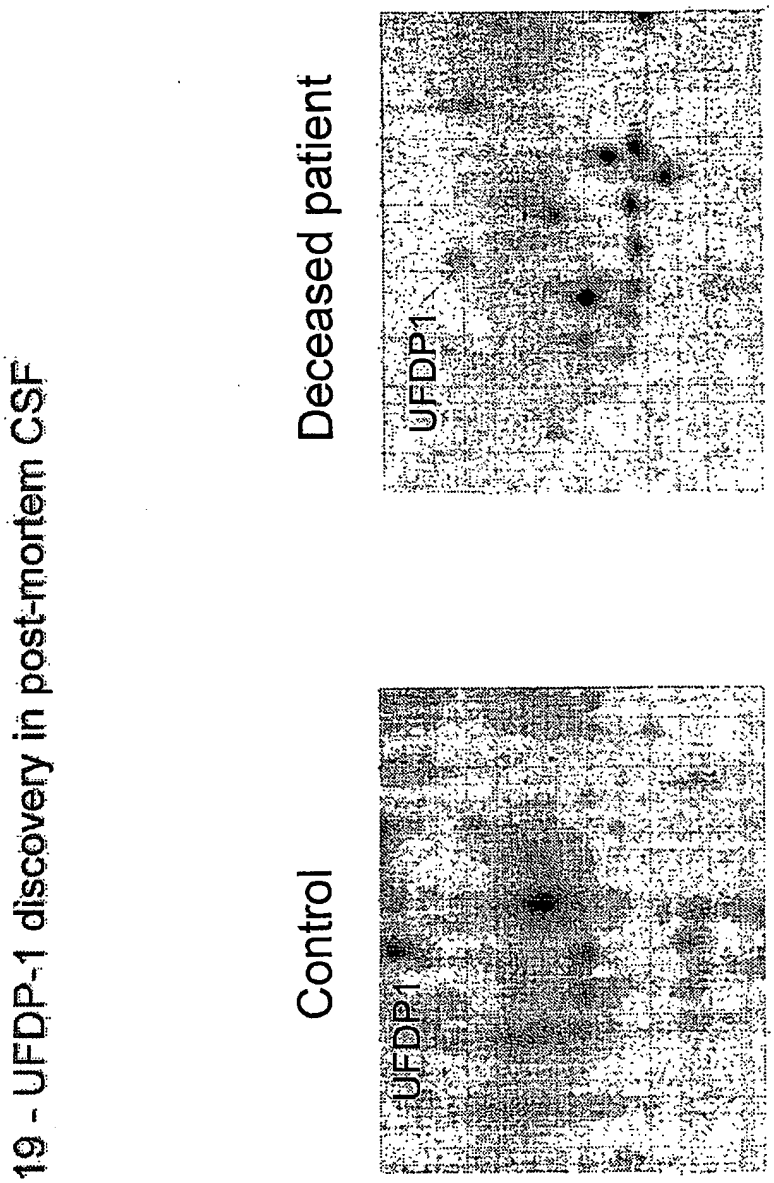
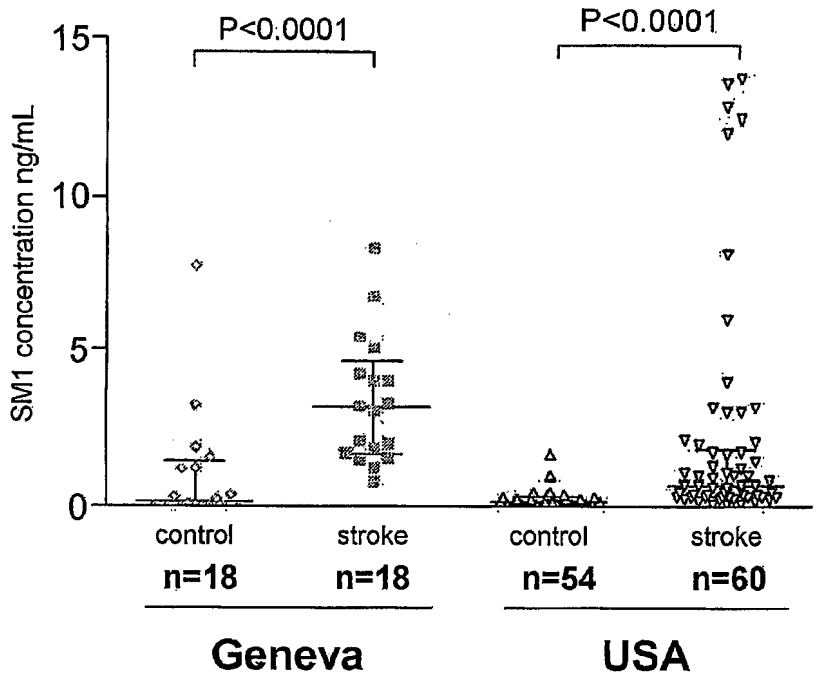


Figure 19 - UFDP-1 discovery in post-mortem CSF

Figure 20 - UFDP1 plasma concentration: ELISA



	Geneva	USA
sensitivity	94.4%	86.6%
specificity	78%	70%

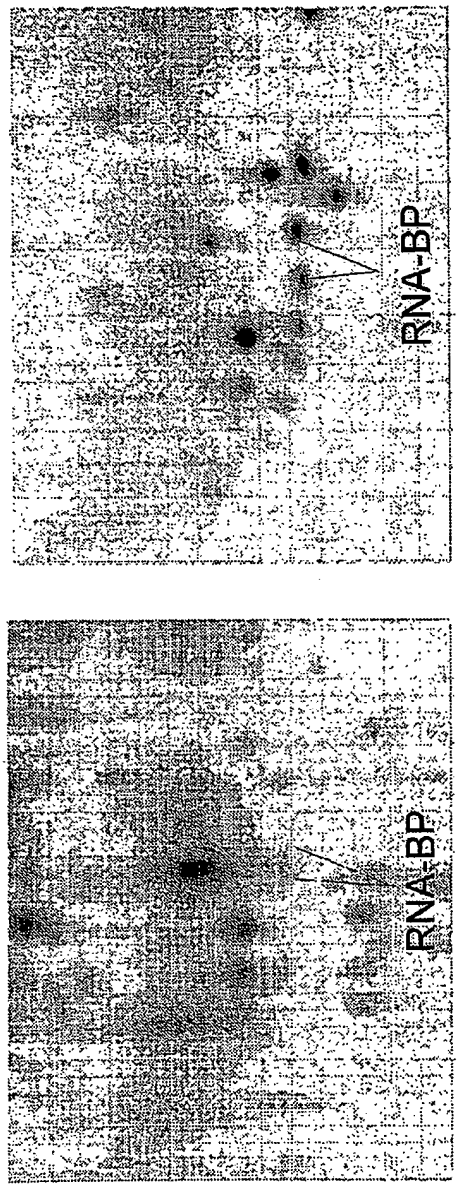
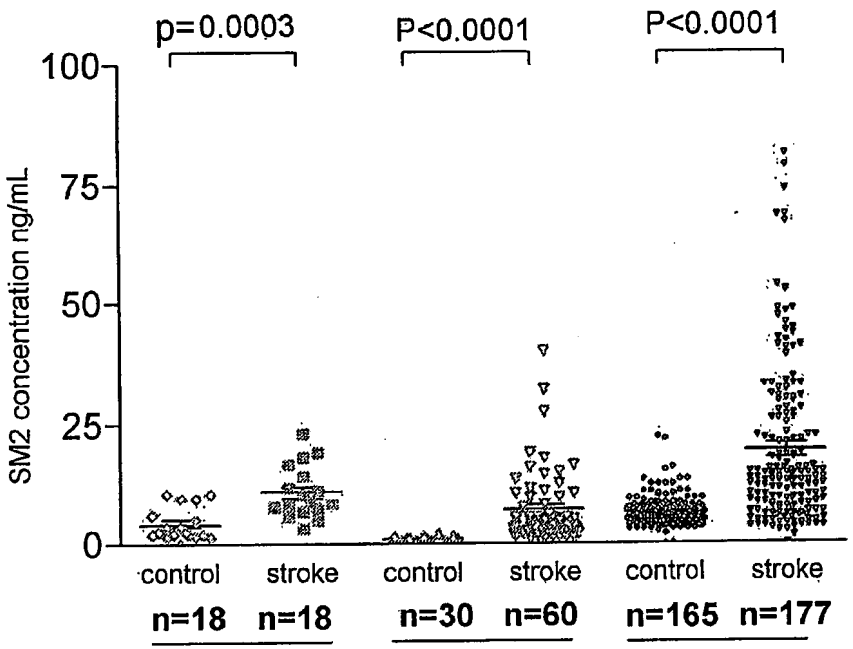


Figure 21 - RNA-BP discovery in post-mortem CSF

Figure 22 - RNA-BP plasma concentration: ELISA



	Study 1	Study 2	Study 3
sensitivity	94.4%	93%	79.6%
specificity	72.2%	83.3%	60%

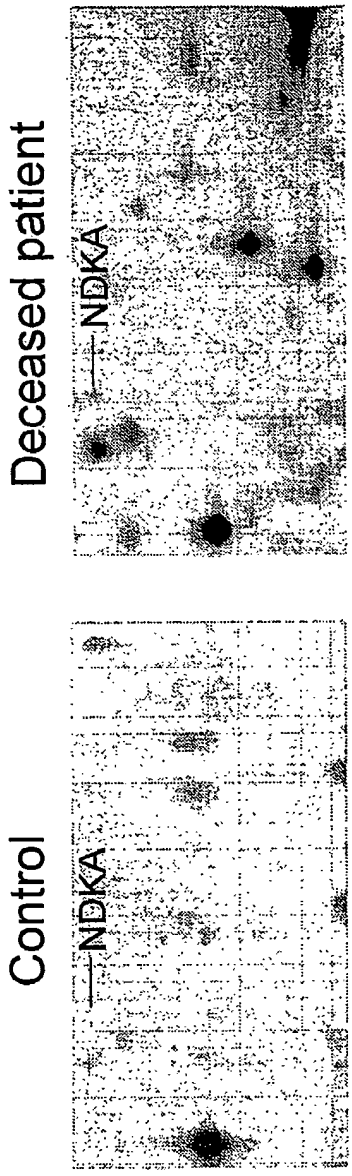
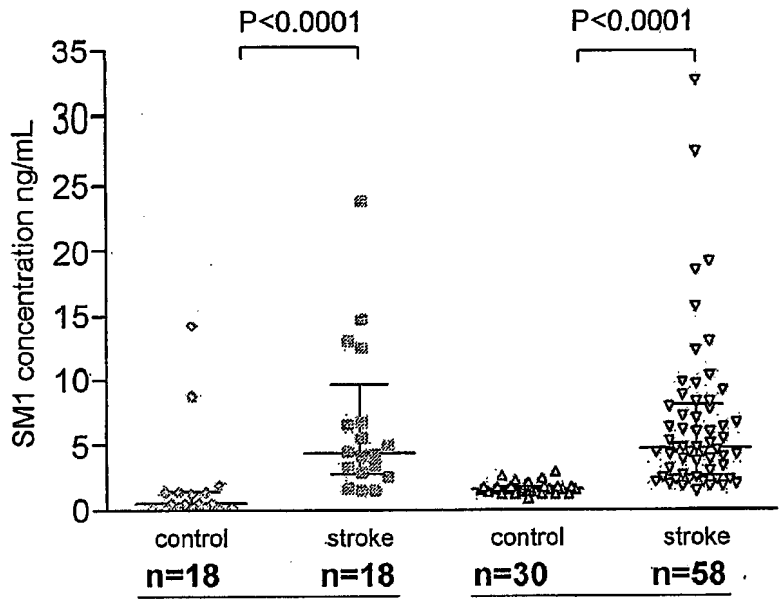


Figure 23 - NDKA discovery in post-mortem CSF

Figure 24 - NDKA plasma concentration: ELISA



	Geneva	USA
sensitivity	100%	100%
specificity	83.3%	72.4%

Figure 25a - Time onset of symptoms

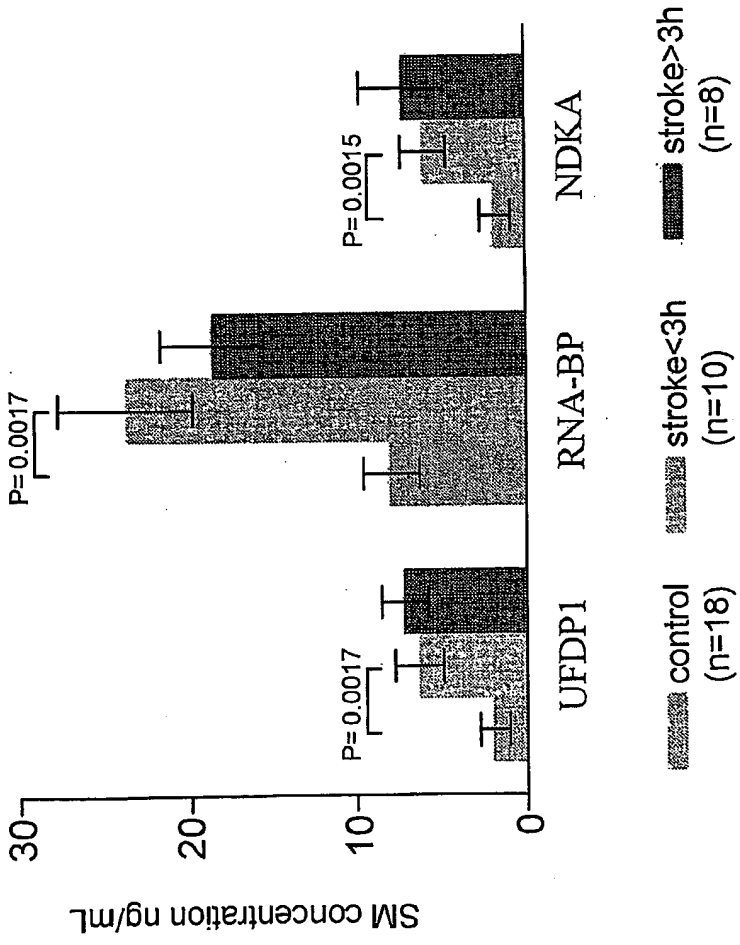
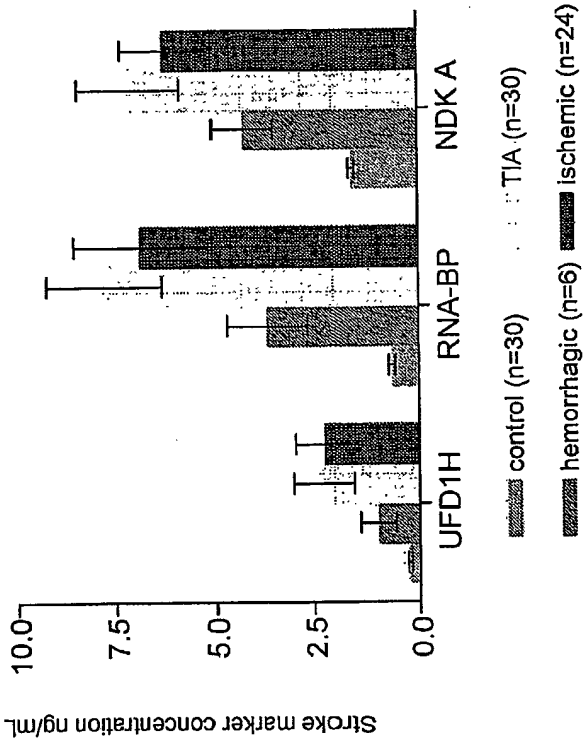


Figure 25b - Type of stroke

(USA data, mean±SEM, Mann Whitney test)

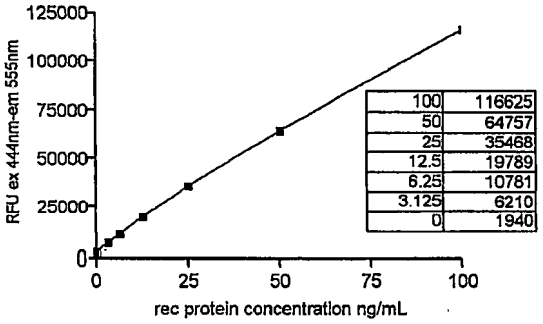


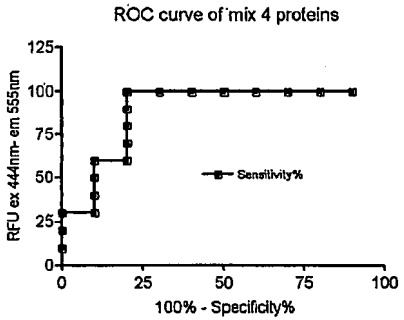
control vs. hemorrhagic $p<0.05$
control vs. TIA $p<0.001$
control vs. ischemic $p<0.001$

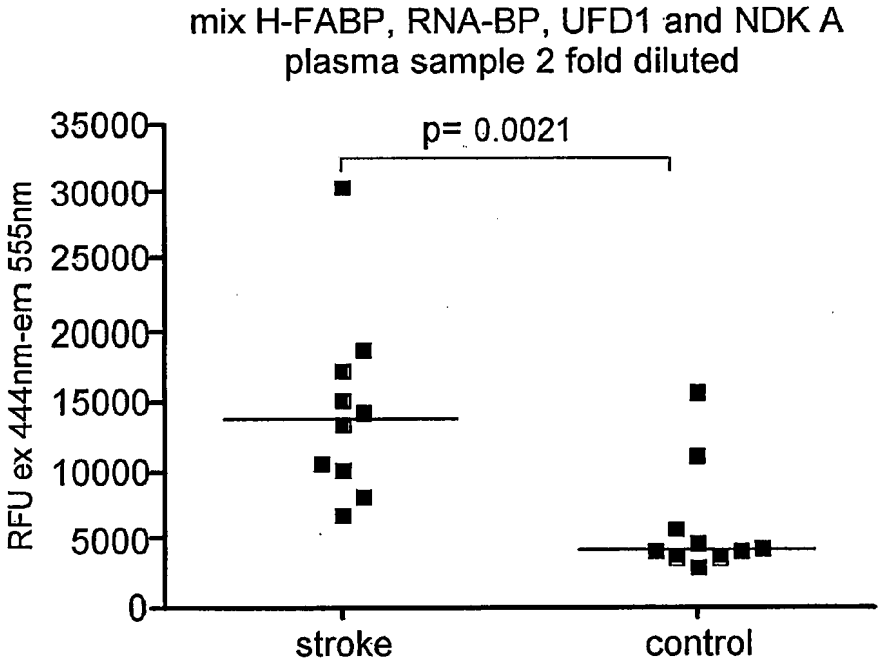
Figure 26 - PANEL of early plasmatic markers of stroke

Protein	Marker type	Sensitivity %	Specificity %
H-FABP	Early diagnosis marker of stroke	68	100
UFDP1	Early diagnosis marker of stroke	94	78
RNA-BP	Early diagnosis marker of stroke	94	72
NDKA	Early diagnosis marker of stroke	100	83

Figure 27. Mix of UFD1, RNA-BP, NDK A and H-FABP in the same well. Detection of the total signal generated by all the proteins in new plasma samples 2 fold diluted. Antibodies sandwich immunofluorescent ELISA. Crude values kinetic fluorescent mode. Controls/stroke matched age/sex







	P (Mann et Whitney)	SE	SP
S vs. C	0.0021	100%	80%

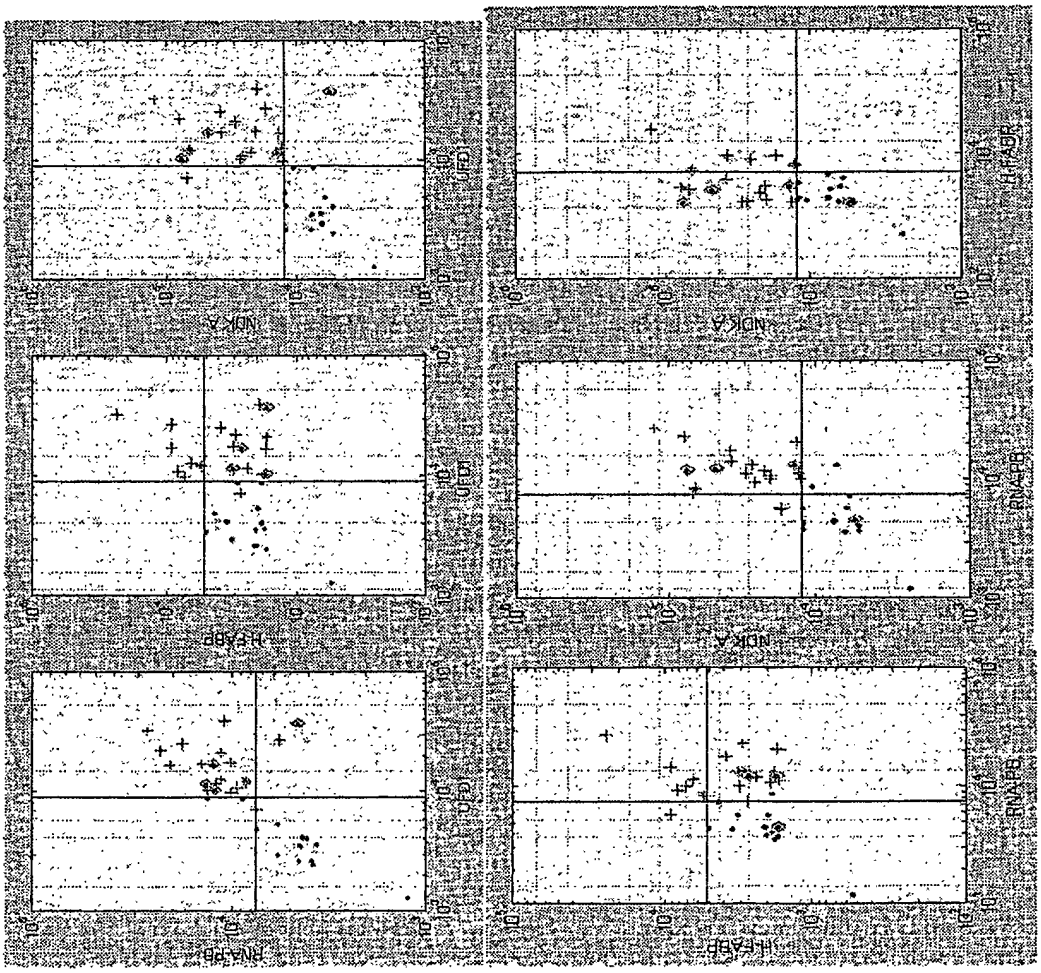


Figure 28. Graphic representation of combination of 2 out the 4 biomarkers of interest. Indicated cut-off (horizontal and vertical lines) are the ones given by us. Dot: negative controls, cross, stroke patients, dots in diamonds: false positive control samples.

FIGURE 29A

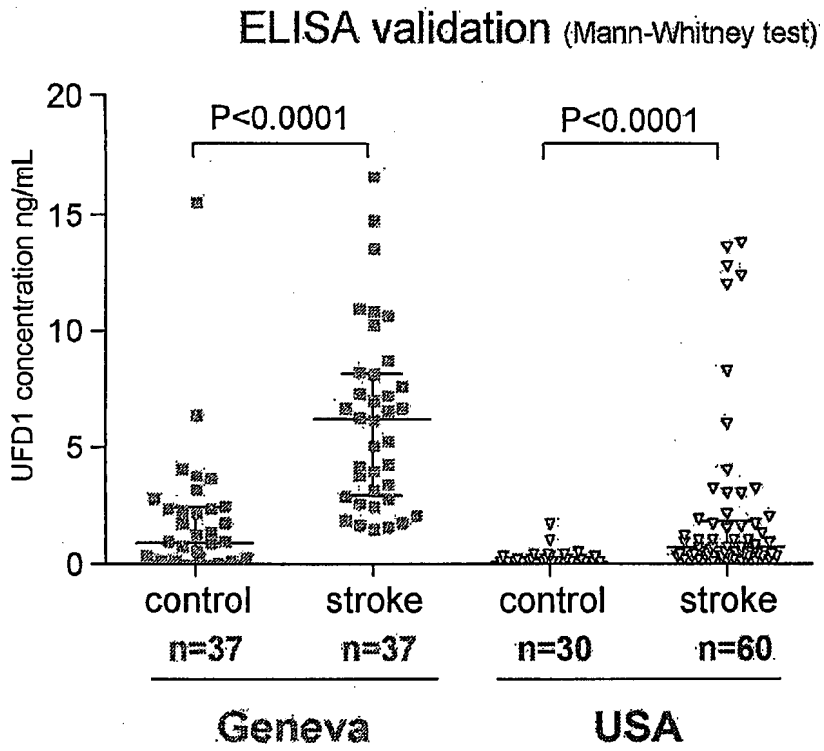
Patient number	Diag	Sex	year of birth (year)	time onset of symptoms (min)	UFD1 (ng/mL)	RNA-BP (ng/mL)	NDK A (ng/mL)
186	I	M	1931	30	1.67	9.58	1.73
253	I	F	1975	45	16.76	15.84	3.01
245	I	M	1925	75	3.42	13.14	6.73
243	H	M	1938	78	8.11	36.64	4.15
239	TIA	M	1923	100	6.31	22.38	6.60
202	H	M	1949	75	4.04	11.26	12.52
229	H	M	1932	125	2.65	23.43	NAN
271	I	M	1913	127	3.77	17.96	3.50
256	TIA	F	1935	180	6.59	14.43	3.16
267	I	M	1928	180	10.27	28.55	13.13
208	I	F	1945	480	4.22	16.95	1.61
212	I	M	1934	630	11.04	6.29	2.46
258	I	M	1920	1440	6.17	33.71	4.27
234	TIA	M	1914	2880	8.70	38.62	14.93
246	I	M	1920	2880	3.18	20.36	4.47
250	I	M	1908	5760	13.61	46.21	24.02
240	I	M	1926	7200	2.91	14.42	1.56
254	I	F	1960	NAN	8.18	16.70	5.02
249	I	M	1931	720	NAN	NAN	5.60
255	I	M	1910	2880	7.31	47.38	4.41
298	I	M	1910	225	7.58	55.55	32.84
154	I	F	1910	165	6.72	13.22	7.73
179	I	F	1912	150	6.74	13.62	4.08
248	TIA	F	1912	150	10.72	19.00	4.59
225	I	M	1915	1440	4.35	13.74	12.98
156	I	F	1919	650	1.87	4.87	0.84
173	I	M	1920	2880	7.00	13.00	5.92
205	I	M	1920	2880	10.94	14.83	6.12
299	I	F	1923	2880	7.19	26.47	31.49
245	I	M	1925	75	2.83	9.61	7.00
189	TIA	M	1926	360	2.07	8.68	2.54
181	TIA	M	1930	70	1.60	3.98	0.95
176	I	M	1932	2880	5.34	10.88	2.24
135	I	F	1933	275	14.85	18.60	6.38
161	I	M	1936	135	1.83	11.60	NAN
285	I	M	1938	240	2.48	9.92	NAN
215	TIA	M	1933	715	1.54	6.05	NAN
235	I	M	1970	195	5.12	16.09	NAN
368	ctrl	M	1931	NAN	3.17	18.48	14.26
401	ctrl	F	1972	NAN	0.00	0.00	0.00
404	ctrl	M	1925	NAN	0.02	4.10	0.46
388	ctrl	M	1938	NAN	0.00	2.88	1.37
464	ctrl	M	1923	NAN	0.00	6.42	0.23
305	ctrl	M	1949	NAN	15.62	4.22	0.06
317	ctrl	M	1931	NAN	2.47	20.53	0.41

FIGURE 29B

Patient number	Diag	Sex	year of birth (year)	time onset of symptoms (min)	UFD1 (ng/mL)	RNA-BP (ng/mL)	NDK A (ng/mL)
439	ctrl	M	1913	NAN	0.34	3.78	1.37
378	ctrl	F	1935	NAN	0.00	3.91	0.48
339	ctrl	M	1929	NAN	0.00	2.73	-0.02
349	ctrl	F	1946	NAN	0.26	3.32	0.00
379	ctrl	M	1934	NAN	2.44	11.95	1.09
400	ctrl	M	1922	NAN	0.65	9.46	0.15
322	ctrl	M	1915	NAN	0.86	6.39	1.42
443	ctrl	M	1919	NAN	3.74	20.73	1.76
450	ctrl	M	1909	NAN	6.42	18.62	8.71
430	ctrl	M	1926	NAN	0.00	4.07	0.48
354	ctrl	F	1955	NAN	0.09	2.47	0.24
389	ctrl	M	1909	NAN	2.78	9.08	3.02
371	ctrl	M	1910	NAN	1.30	4.70	0.00
352	ctrl	F	1911	NAN	1.46	5.54	0.01
376	ctrl	F	1912	NAN	0.00	2.58	0.00
429	ctrl	F	1912	NAN	2.45	5.68	0.00
399	ctrl	M	1916	NAN	0.46	6.28	0.61
434	ctrl	F	1919	NAN	2.22	5.94	0.25
459	ctrl	M	1921	NAN	0.88	3.75	0.43
462	ctrl	M	1921	NAN	0.41	2.16	0.00
444	ctrl	F	1922	NAN	4.13	5.52	0.14
468	ctrl	M	1923	NAN	1.80	5.14	2.22
386	ctrl	M	1927	NAN	0.98	2.96	0.39
397	ctrl	M	1931	NAN	2.30	16.58	0.12
402	ctrl	M	1933	NAN	3.86	7.32	0.15
416	ctrl	F	1934	NAN	0.00	2.13	0.45
307	ctrl	M	1936	NAN	0.19	3.08	NAN
321	ctrl	M	1938	NAN	0.23	2.07	NAN
417	ctrl	M	1943	NAN	1.83	10.41	NAN
377	ctrl	M	1966	NAN	1.05	8.59	NAN

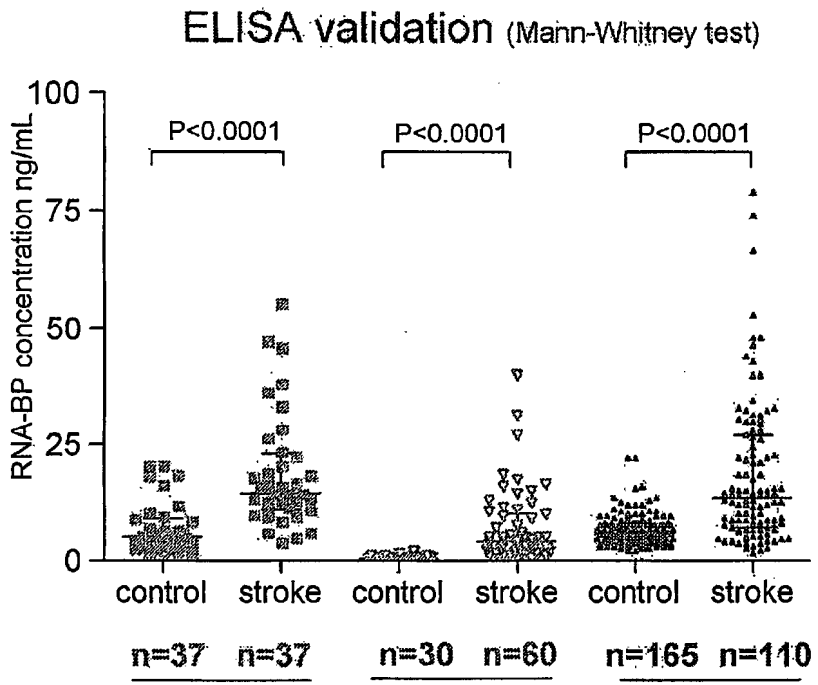
NAN : not tested

Figure 30: Ubiquitin Fusion Degradation Protein



	Geneva	USA
sensitivity	91.9%	86.6%
specificity	67.5%	70%

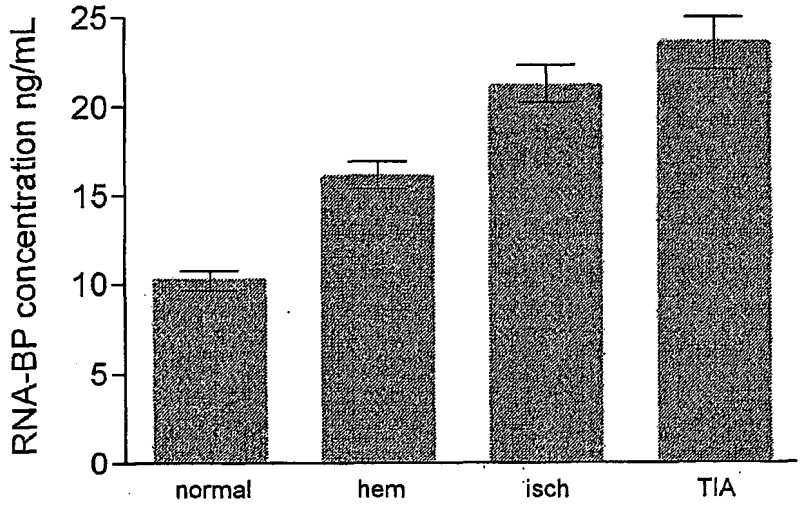
Figure 31: RNA-Binding Protein in plasma



	Geneva	USA1	USA2
sensitivity	86.5%	93%	79.6%
specificity	81.1%	83.3%	60%

Figure 32: RNA-binding protein in plasma (USA-3)

Large-scale study USA data on 633 patients

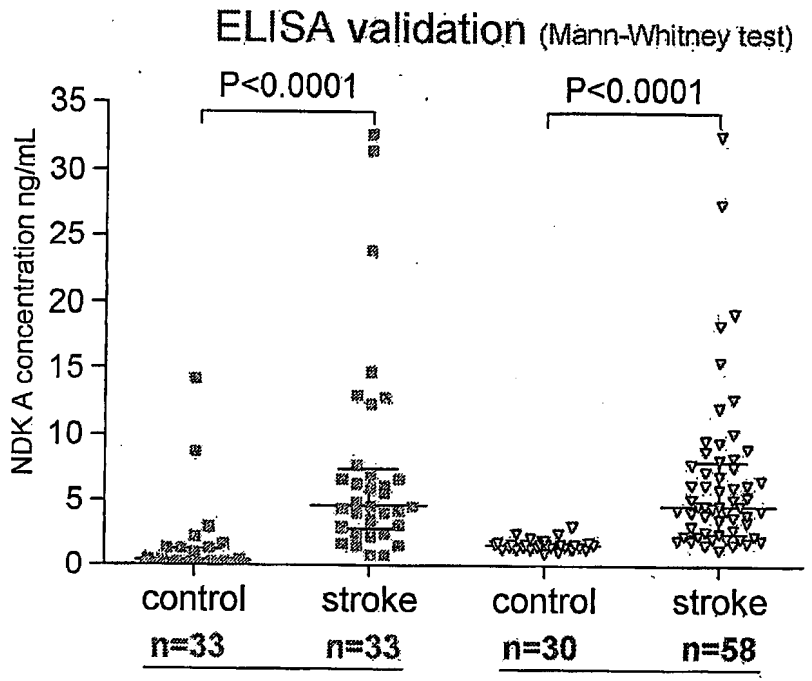


	normal	hem	isch	TIA
n=	100	226	183	124
Mean	10.1	16.1	21.3	23.5
Std. Deviation	5.10	11.9	14.6	16.0
Std. Error	0.510	0.791	1.08	1.44

Figure 33: RNA-binding protein in plasma (USA-3)

Kruskal-Wallis statistic	79.78			
Dunn's Multiple Comparison Test	P value	CO	SE	SP
normal vs hem	P < 0.001	9.5	68	62
normal vs isch	P < 0.001	9.5	77.6	62
normal vs TIA	P < 0.001	9.5	81.4	62
hem vs isch	P < 0.01	13.5	60	54.9
hem vs TIA	P < 0.001	15.95	64.5	63.7
isch vs TIA	P > 0.05			

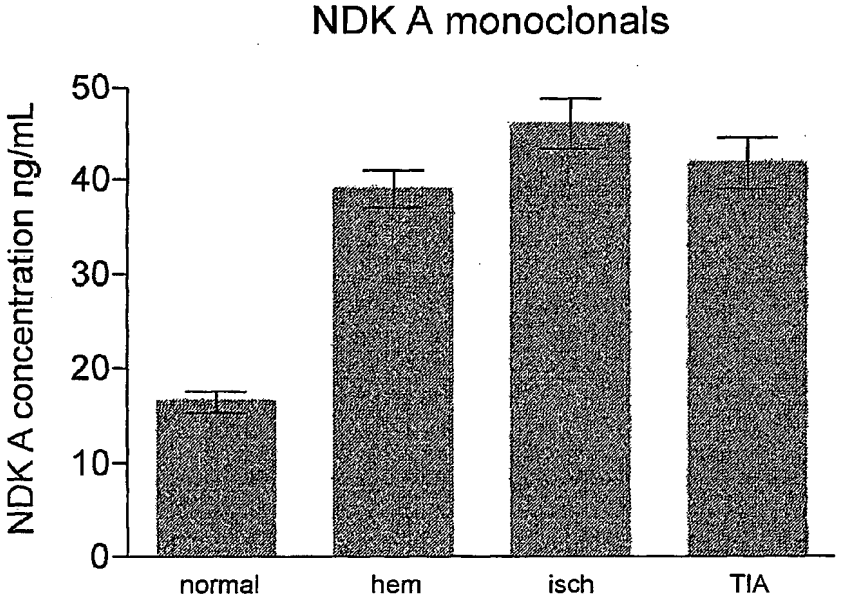
Figure 34: Nucleoside Diphosphate Kinase A



	Geneva	USA
sensitivity	93.9%	100%
specificity	84.8%	72.4%

Figure 35: Nucleoside diphosphate kinase A (USA-3)

Large-scale study USA data on 622 patients



	normal	hem	isch	TIA
n=	99	221	183	119
Mean	16.4	38.9	45.8	41.6
Std. Deviation	11.6	30.0	37.6	30.0
Std. Error	1.17	2.02	2.78	2.75

Figure 36: Nucleoside diphosphate kinase A (USA-3)

Kruskal-Wallis statistic 109.9				
Dunn's Multiple Comparison Test	P value	CO	SE%	SP%
normal vs hem	P < 0.001	18	81	76.7
normal vs isch	P < 0.001	18	74.8	76.7
normal vs TIA	P < 0.001	18	81.5	76.7
hem vs isch	P > 0.05			
hem vs TIA	P > 0.05			
isch vs TIA	P > 0.05			

Figure 37: Time onset of symptoms

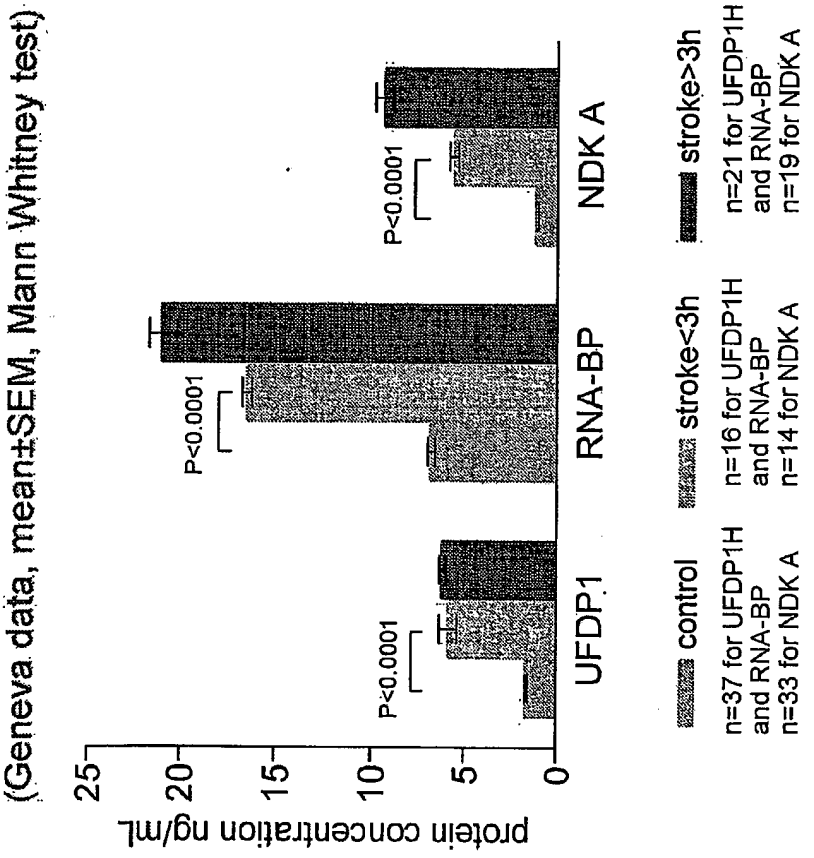


Figure 38: Type of stroke